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REVIEW

Ferroptosis in Hepatocellular Carcinoma: Mechanisms and Targeted Therapy

Amir Ajoolabady ¹, Daolin Tang ^{2*}, Guido Kroemer ^{3,4,5*}, and Jun Ren ^{1,6*}

¹ Shanghai Institute of Cardiovascular Diseases, Department of Cardiology, Zhongshan Hospital Fudan University, Shanghai, China; ² Department of Surgery, UT Southwestern Medical Center, Dallas, TX, 75390, USA; ³ Centre de Recherche des Cordeliers, Equipe labellisée par la Ligue contre le cancer, Université de Paris, Sorbonne Université, Inserm U1138, Institut Universitaire de France, Paris, France; ⁴ Metabolomics and Cell Biology Platforms, Institut Gustave Roussy, Villejuif, France; ⁵ Pôle de Biologie, Hôpital Européen Georges Pompidou, AP-HP, Paris, France; ⁶ Department of Laboratory Medicine and Pathology, University of Washington, Seattle, WA 98195 USA.

Correspondence to:

Prof. Jun Ren (jren@uw.edu)

Prof. Guido Kroemer (kroemer@orange.fr)

Prof. Daolin Tang (daolin.tang@utsouthwestern.edu)

20 **Abstract**

21 Hepatocellular carcinoma is the most prevalent form of primary liver cancer with a
22 multifactorial etiology comprising genetic, environmental, and behavioral factors. Evading cell
23 death is a defining hallmark of hepatocellular carcinoma, underpinning tumor growth, progression,
24 and therapy resistance. Ferroptosis is a form of nonapoptotic cell death driven by an array of
25 cellular events, including intracellular iron overload, free radical production, lipid peroxidation,
26 and activation of various cell death effectors, ultimately leading to rupture of plasma membrane.
27 Although induction of ferroptosis is an emerging strategy to suppress hepatocellular carcinoma,
28 malignant cells manage to develop adaptive mechanisms, conferring resistance to ferroptosis and
29 ferroptosis-inducing drugs. Herein, we aim at elucidating molecular mechanisms and signaling
30 pathways involved in ferroptosis and offer our opinions on druggable targets and new therapeutic
31 strategy in an attempt to restrain growth and progression of hepatocellular carcinoma through
32 induction of ferroptotic cell death.

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34 **Keywords:** *Ferroptosis, cell death, hepatocellular carcinoma, NRF2*

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41 **Abbreviations**

42 ABCA8/9 (ATP binding cassette subfamily A member 8/9), ABCB6 (ATP binding cassette
43 subfamily B member 6), ABCC5 (ATP binding cassette subfamily C member 5), ACSL4 (acyl-
44 CoA synthetase long chain family member 4), AIFM2 (apoptosis inducing factor mitochondria
45 associated 2), AKR1B1 (aldo-keto reductase family 1 member B), AKR1B10 (aldo-keto reductase
46 family 1 member B10), AKR1C1/2/3 (aldo-keto reductase family 1 member C1/2/3), AKT1 (AKT
47 serine/threonine kinase 1), ALDH1A1 (aldehyde dehydrogenase 1 family member A1), ALOXs
48 (arachidonate lipoxygenase enzymes), ALOXE3 (arachidonate lipoxygenase 3), ALOX5
49 (arachidonate 5-lipoxygenase), ALOX12B (arachidonate 12-lipoxygenase, 12R type), ALOX12
50 (arachidonate 12-lipoxygenase, 12S type), ALOX15B (arachidonate 15-lipoxygenase type B),
51 ALOX15 (arachidonate 15-lipoxygenase), AMPK (AMP-activated protein kinase), ARE
52 (antioxidant response element), ARF (ADP ribosylation factor 1), ARNTL (aryl hydrocarbon
53 receptor nuclear translocator like), ATF4 (activating transcription factor 4), BAP1 (BRCA1
54 associated protein 1), BECN1 (beclin 1), bTrCP (beta-transducin repeat containing E3 ubiquitin
55 protein ligase), BLVRA/B (biliverdin reductase A/B), CHAC1 (ChaC glutathione specific
56 gamma-glutamylcyclotransferase 1), CHOP (C/EBP homologous protein), CISD1 (CDGSH iron
57 sulfur domain 1), CISD2 (CDGSH iron sulfur domain 2), CLTRN (collectrin, amino acid transport
58 regulator), COMMD10 (COMM domain containing 10), CoQ10 (coenzyme Q10), CUL3 (cullin
59 3), CYBRD1 (cytochrome B reductase 1), CYP2D6 (cytochrome P450 family 2 subfamily D
60 member 6), DHODH (dihydroorotate dehydrogenase (quinone)), DLD (dihydrolipoamide
61 dehydrogenase), DMT1 (divalent metal transporter 1), DPP4 (dipeptidyl peptidase 4), DRAM1
62 (DNA damage regulated autophagy modulator 1), E2F1 (E2F transcription factor 1), eIF2 α
63 (eukaryotic translation initiation factor 2 subunit alpha), EGF (epidermal growth factor), EGFR

64 (epidermal growth factor receptor), EGLN2 (Egl-9 family hypoxia inducible factor 2), EMP1
65 (epithelial membrane protein 1), ESCRT III (endosomal sorting complex required for transport),
66 ETS1 (ETS proto-oncogene 1, transcription factor), FAM134B (family with sequence similarity
67 134, member B), FAT (fatty acid translocase), FATP (fatty acid transport protein), FECH
68 (ferrochelatase), FSP1 (ferroptosis suppressor protein 1), FTH1 (ferritin heavy chain 1), FTL
69 (ferritin light chain), G6PD (glucose-6-phosphate dehydrogenase), GABPB1 (GA-binding protein
70 subunit beta-1), GABPB1-AS1 (GABPB1 antisense RNA 1), GCH1 (GTP cyclohydrolase 1),
71 GCL (glutamate-cysteine ligase), GCLC (glutamate-cysteine ligase catalytic subunit), GCLM
72 (glutamate-cysteine ligase modifier subunit), GCN2 (general control nonderepressible 2), GPX7
73 (glutathione peroxidase 7), GSR (glutathione-disulfide reductase), GSS (glutathione synthetase),
74 GSTs (glutathione S-transferases), GSTA1 (glutathione s-transferase alpha 1), GSTP1
75 (glutathione s-transferase Pi 1), GSTZ1 (glutathione S-transferase zeta 1), HIF1- α (hypoxia-
76 inducible factor 1-alpha), HMGB1 (high mobility group box 1), HMGCR (3-hydroxy-3-
77 methylglutaryl-CoA reductase), HMOX1 (heme oxygenase 1), HSP90AB1 (heat shock protein 90
78 alpha family class B member 1), HSPA5 (heat shock protein family A (Hsp70) member 5), HSPA8
79 (heat shock protein family A (Hsp70) member 8), IREB2 (iron responsive element binding protein
80 2), Keap1 (kelch-like ECH-associated protein 1), LC3B/MAP1LC3B (microtubule associated
81 protein 1 light chain 3 beta), LAMP2 (lysosomal associated membrane protein 2), LDH (lactate
82 dehydrogenase), LPCAT3 (lysophosphatidylcholine acyltransferase 3), MAF [v-maf
83 musculoaponeurotic fibrosarcoma oncogene homolog (avian)], MafG (v-maf avian
84 musculoaponeurotic fibrosarcoma oncogene homolog G), METTL14 (methyltransferase 14, N6-
85 adenosine-methyltransferase subunit), MT1G (metallothionein 1G), NADPH
86 (dihyronicotinamide-adenine dinucleotide phosphate), NCOA4 (nuclear receptor coactivator 4),

87 NRF2 (nuclear factor erythroid 2-related factor 2), NOXs (NADPH oxidases), NOX4 (NADPH
88 oxidase 4), NQO1 (NAD(P)H quinone dehydrogenase 1), NROB2 (nuclear receptor subfamily 0
89 group B member 2), NUPR1 (nucleoprotein 1), p21/CDKN1A (cyclin-dependent kinase inhibitor
90 1), p62 (sequestosome-1), PABPC1 (poly(A) binding protein cytoplasmic 1), PCBP1 (poly(RC)
91 binding protein 1), PCBP2 (poly(rC) binding protein 2), PD-1 (programmed cell death protein 1),
92 PD-L1 (programmed death-ligand 1) PERK (protein kinase R (PKR)-like endoplasmic reticulum
93 kinase), PI3K (phosphoinositide 3-kinase), PLA2G2A (phospholipase A2 group IIA), PPARG
94 (peroxisome proliferator activated receptor gamma), PRDX1/5/6 (peroxiredoxin 1/5/6), PROM2
95 (prominin 2), PSTK (phosphoseryl-TRNA kinase), QSOX1 (quiescin sulfhydryl oxidase 1), RAN
96 (RAN, Member RAS Oncogene Family), RAB7A (RAB7A, member RAS oncogene family),
97 RBX1 (ring-box 1), RIPK1 (receptor interacting serine/threonine kinase 1), Se (selenium),
98 SLC1A5 (solute carrier family 1 member 5), SLC3A2 (solute carrier family 3 member 2),
99 SLC7A10 (solute carrier family 7 member 10), SLC7A11 (solute carrier family 7 member 11),
100 SLC11A2 (solute carrier family 11 member 2), SLC25A28 (solute carrier family 25 member 28),
101 SLC25A37 (solute carrier family 25 member 37), SLC39A8 (solute carrier family 39 member 8),
102 SLC39A14 (solute carrier family 39 member 14), SLC40A1 (solute carrier family 40 member 1),
103 SLC48A1 (solute carrier family 48 member 1), sMaf (small Maf protein), SPARC (secreted
104 protein acidic and cysteine rich), STEAP3 (STEAP3 metalloreductase), STAT3 (signal transducer
105 and activator of transcription 3), TAZ (tafazzin, phospholipid-lysophospholipid transacylase),
106 TEAD1 (transcriptional enhancer factor TEF-1), TFR1 (transferrin receptor 1), TNF- α (tumour
107 necrosis factor alpha), TP53/p53 (tumor protein p53), TXNRD1 (thioredoxin reductase 1), YAP1
108 (yes1 associated transcriptional regulator), YTHDF2 (YTH N6-methyladenosine RNA binding
109 protein 2).

110 **1. Introduction**

111 Hepatocellular carcinoma (HCC) is the primary form of liver malignancy with the highest
112 prevalence in patients with chronic liver disease ¹. Liver cancer ranks the 3rd among all cancer
113 mortalities globally next to lung and colorectal cancers and HCC constitutes 75% of liver cancers
114 ². The 5-year survival rate for localized HCC is 32.6%, compared with 10.8% and 2.4% for
115 regional and metastatic HCC, respectively, underscoring the necessity of novel HCC treatment
116 options ³. **Chronic hepatitis** “(see the Glossary)” resulting from persistent infections with
117 hepatotropic viruses often ending in HCC ⁴. Moreover, the incidence of HCC caused by metabolic
118 stress is believed to rise dramatically in comparison with virus-induced HCC owing to worldwide
119 pandemics of obesity, diabetes mellitus, and metabolic diseases ^{5,6}. Indeed, **non-alcoholic**
120 **steatohepatitis (NASH)** is now considered the main risk factor of HCC in developed countries ⁷.
121 In the United States, there are 34,800 HCC cases associated with NASH each year, and this number
122 is projected to reach a toll of 44,800 annual cases by 2025 ³. Considering the high prevalence of
123 HCC worldwide, it is pertinent to elucidate HCC pathogenesis in sufficient details to eventually
124 identify useful therapeutic approaches ⁸. The main goal of targeted therapy is to kill cancer cells
125 without harming otherwise healthy cells. Selective induction of distinct cell death is a possible
126 strategy to eliminate malignant hepatocytes ^{9,10}. Cell death can be categorized into either accidental
127 or regulated ¹¹. Accidental cell death displays no defined molecular mechanisms, regulators, or
128 signals, whereas regulated cell death (RCD) is characterized by tightly regulated molecular and
129 signaling machineries ¹². Reactive oxygen species (ROS) act as the prime drivers of RCD under
130 impaired cellular antioxidant systems ¹³. Broadly speaking, ferroptosis refers to a form of ROS-
131 mediated RCD, which is executed by dynamic signaling cascades (**Figs 1, 2, 3**) ¹⁴. Ferroptosis is
132 instigated by intracellular iron accumulation and subsequent ROS generation, resulting in lipid

133 peroxidation and membrane disruption, which ignites an array of downstream cell signal pathways
134 and activates suicidal effector proteins (**Figs 1, 2, 3**)¹⁵. A plethora of studies have demonstrated
135 that ferroptosis abrogates growth and proliferation of HCC cells in *in vitro* and *in vivo* xenograft
136 models^{16,17}. However, HCC cells may develop adaptive/protective machineries to counteract
137 ferroptosis, thus maintaining tumor growth. Specific molecules that determine the susceptibility to
138 ferroptosis have been identified. Here, we aim at deciphering possible mechanisms underpinning
139 ferroptotic cell death or ferroptosis resistance in HCC cells, as well as at highlighting targetable
140 components and potential therapeutics in the clinical management of HCC.

141

142 **2. Iron metabolism and its linkage with molecular mechanisms of ferroptosis**

143 Iron is a vital trace element for all living organisms and exists in two major forms in human
144 body, namely, ferrous iron (Fe^{2+} , absorbable) and ferric iron (Fe^{3+} , less soluble)^{18,19}. Iron
145 metabolism is a complex process involving four main phases: absorption, distribution, storage, and
146 excretion (**Fig 1**)²⁰. Both endogenous and exogenous (nutritional) sources supply the iron needed
147 for maintaining homeostasis²¹. Duodenal mucosal cells absorb nutritional iron to either release it
148 into circulation or store as ferritin (**Fig 1**)²⁰. In conditions of iron deficiency, uptake of exogenous
149 iron and utilization of endogenous iron rises, while ferritin storage plummets. On the other hand,
150 in conditions of iron sufficiency, intestinal iron absorption declines, while ferritin storage
151 increases, in an effort to avoid excessive iron accumulation²². Transferrin is a plasma glycoprotein
152 that binds and transports circulatory Fe^{3+} to various organs²³. Serum transferrin-bound Fe^{3+} is
153 discerned by TFR1 receptors (encoded by *TFRC*) on cell membranes, mediating endosomal
154 delivery of Fe^{3+} into cytosols (**Figs 1, 2**)²⁴. Subsequently, Fe^{3+} is reduced to Fe^{2+} by endosomal
155 STEAP3 enzyme, prior to cytoplasmic release via SLC11A2 transporters (**Fig 1**)²⁵. Cytosolic Fe^{2+}

156 supplies multiple biological functions, including DNA biosynthesis, oxygen transport, and
157 regulation of metabolic pathways. Ferritin, a cytosolic protein that consists of two subunits (FTL
158 and FTH1), is the primary protein for iron storage (in the form of Fe^{3+})²⁶. FTH1 and FTL share
159 high sequence homology albeit they differ in iron utilization and storage²⁶. Importantly,
160 mammalian cells employ a unique cellular mechanisms termed ferritinophagy, a selective form of
161 **autophagy** that facilitates the degradation of ferritin, to liberate Fe^{2+} from its carrier (**Figs 1, 3, 4**)
162 ²⁷. Excessive intracellular Fe^{2+} can be extruded by the carrier SLC40A1 towards extracellular
163 space, where it is converted back into Fe^{3+} , to balance redox state and iron homeostasis (**Figs 1, 2**)
164 ^{28,29}. In theory, each step of this dynamic process might be therapeutically targeted for combating
165 iron-related diseases, as well as excessive or deficient ferroptosis. Despite the availability of
166 transferrin-bound iron, the majority of mammalian cells possess a feature of uptaking non-
167 transferrin-bound iron (NTBI)³⁰. Under conditions of iron overload (e.g., **hemochromatosis**) iron
168 load may exceed the transferrin carrying capacity, thereby, floating in circulation as NTBI³¹. It is
169 thought that cell surface **ferrireductases** such as CYBRD1 and cell-secreted reductants such as
170 ascorbate mediate the uptake of NTBI³¹. Mechanistically, ferrireductases and ascorbate convert
171 NTBI into Fe^{2+} , which is subsequently imported into the cytosol via transporters including
172 SLC39A14, SLC39A8, and SLC11A2 (**Fig 1**)³². Ensuing erastin challenge (a ferroptosis inducer),
173 expression of *SLC11A2* and *TFRC* was upregulated in HT1080 cells³³, indicating contribution of
174 NTBI and transferrin-bound iron in ferroptosis induction. Similarly, liver-specific *SLC39A14*
175 knockout inhibited ferroptosis in mouse livers³⁴, suggesting a role for NTBI in ferroptosis
176 induction. Thus, inhibition of its transport may reverse ferroptosis. Despite obscurity in iron
177 metabolic anomalies in the induction of ferroptosis, ample evidence has depicted compelling
178 evidence of iron metabolic involvement in ferroptosis³⁵. For instance, (i) inhibition and prevention

179 of ferroptotic cell death with iron chelators both *in vivo* and *in vitro* ³⁵, (ii) ferroptosis induction is
180 accompanied by elevated intracellular labile iron ³⁶, and (iii) iron supplementation enhanced
181 ferroptosis induction upon treatment with erastin ³⁷. Hence, it is sensible to assume that regulators
182 of iron storage, influx, efflux, and utilization might be involved in the regulation of ferroptosis.
183 The scenario behind iron metabolic anomaly and linkage to ferroptosis begins with excessive
184 extracellular or intracellular iron prompting iron overload, resulting in constitutive oxidative stress
185 and organ damage ^{38,39}. Iron overload triggers ferroptosis through two mechanisms, namely (i)
186 excessive ROS generation culminating in oxidative damage of lipids and DNA, and (ii) activation
187 of lipoxygenases and other enzymes containing non-heme iron, resulting in lipid peroxidation
188 (**Figs 1, 2, 3**) ⁴⁰⁻⁴³. Two remarkable ROS species causing lipid peroxidation are the hydroperoxyl
189 (OOH•) and hydroxyl (OH•) radicals, which are generated by the Fenton reaction resulting from
190 interaction between Fe²⁺ and H₂O₂ (**Figs 1, 2**) ⁴⁴. In this regard, impairment of iron efflux (e.g.,
191 upon *SLC40A1* knockdown) and storage (e.g., upon induction of ferritinophagy or knockdown of
192 either of the two ferritin subunits *FTL* or *FTH1*), as well as elevated iron intake (e.g., due to *TFRC*
193 overexpression) would impose cells at a high risk of ferroptosis ^{29,40-42}. Moreover, iron
194 transportation across lysosomes, mitochondria, and cytosol, as well as activation of IREB2 (an
195 RNA-binding protein regulating iron homeostasis) may influence the organismal or cellular
196 susceptibility to ferroptosis ^{45,46}. In particular, iron overload triggers ferroptosis through excess
197 ROS generation, lipid peroxidation, and many yet undefined signaling molecules and effectors.

198 Upon lipid peroxidation, membrane polyunsaturated fatty acids (PUFAs) are oxidized,
199 thus, dysfunction of cell antioxidant systems may accelerate lipid peroxidation. Therefore,
200 application of synthetic antioxidants including liproxstatin-1 and ferrostatin-1 has been shown to
201 hinder lipid peroxidation ^{47,48}. Lipid peroxidation of phosphatidyl PUFAs (PUFAs-PL) engages

202 specific enzymes, such as ACSL4 and ALOXs (**Figs 1, 2**)^{46,49,50}. ACSL4 catalyzes the synthesis
203 and enrichment of PUFAs, particularly, arachidonic acid in cell membranes, thereby, acting as a
204 pro-ferroptotic factor⁵¹. With regards to the ACSL family, upregulation of ACSL4 is considered
205 a vital biomarker and driver for ferroptotic death in cancer cells⁵¹. Moreover, upregulation of
206 LPCAT3, an essential ACSL4 downstream signal molecule, may foster ferroptosis⁵². ACSL4 and
207 LPCAT3 participate in incorporation of arachidonic acid into cell membranes, thereby, paving the
208 way for ferroptosis induction^{53,54}. For oxidation of PUFAs, several models have been proposed.
209 In the first model, membrane-bound NOXs enzymes catalyze PUFAs oxidation to produce
210 superoxides, thereby, contributing to the build-up of lipid peroxides and ferroptosis induction⁵⁵.
211 In the second model, ALOXs enzymes directly catalyze PUFAs oxygenation to drive ferroptosis
212^{56,57}. Also, cytochrome P450 oxidoreductase plays a cardinal role in ferroptosis of cancer cells by
213 providing ROS for phospholipid peroxidation⁵⁸. Moreover, electron leakage from mitochondrial
214 electron chain can lead to ROS generation and lipid peroxidation⁵⁹. As described above, ACSL4
215 participates in PUFAs-PL production, while ALOXs mediate PUFAs-PL oxidization into
216 hydroperoxy products. The ALOX family includes *ALOX15B*, *ALOX15*, *ALOX12B*, *ALOX12*,
217 *ALOX5*, and *ALOXE3*, all of which participating in ferroptosis in a cell type-or tissue-specific
218 manner^{60,61}. Alternatively, cytochrome P450 oxidoreductase may mediate lipid peroxidation in
219 ferroptosis in an ALOX-independent manner⁶², whereas lipophagy-mediated degradation of lipid
220 droplets provides fatty acids for subsequent lipid peroxidation in HCC cells during ferroptosis⁶².
221 In response to pre-ferroptotic stress, mammalian cells have adapted antioxidant systems, such as
222 antiporter system x_c^- , which exports glutamate to import cysteine into the cell, thus facilitating the
223 synthesis of glutathione (GSH), a major scavenger of intracellular free radicals (**Figs 1, 2**)⁶³.
224 Mechanistically, specific ferroptosis inducers, such as sulfasalazine and erastin ignite ferroptosis

225 via blockade of system xc^- ^{37,64}. Of note, sorafenib, an approved drug for HCC treatment, has been
226 repeatedly evinced to ignite ferroptosis through blockade of system xc^- ^{37,64}. However, controversy
227 exists as sorafenib was reported to be incapable of igniting ferroptosis like erastin and sulfasalazine
228 in a number of tumor cell lines, indicating that sorafenib may not be a *bona fide* inducer of
229 ferroptosis⁶⁵.

230 System xc^- is a transmembrane protein complex containing light chain SLC7A11 and
231 heavy chain SLC3A2 (**Fig 1**). SLC7A11 (a.k.a., xCT) is a transmembrane protein with twelve-
232 pass that is attached to the regulatory single-pass SLC3A2 protein (a.k.a., CD98hc and 4F2hc) via
233 a disulfide bond⁶⁶. It has been evinced that upregulation of SLC7A11-mediated cystine influx can
234 replace the glutathione peroxidase 4 (GPX4)-GSH antioxidant system (described later in the text)
235 in certain malignant cells both *in vivo* and *in vitro*^{67,68}. Besides, upregulation of TP53 has been
236 shown to nullify *SLC7A11* expression, thereby, predisposing to ferroptosis⁶⁹. System xc^- mediates
237 cystine influx into the cytosol where it is chemically reduced to cysteine for GSH synthesis (a low
238 molecular weight antioxidant). GPX4 contains selenocysteine and employs GSH as a cofactor,
239 thereby, mediating reduction of lipid peroxides into non-toxic alcohols. In other words, GPX4
240 reduces lipid-OOH (hydroperoxide) into lipid-OH (alcohol) to retard the production of lipid-O•
241 (alkoxy radicals) from lipid-OOH⁷⁰. Importantly, cells exposed to lipid-generated ROS are under
242 the danger of destruction, as suppressed activity or genetic ablation of *GPX4* was reported to
243 induce massive accumulation of lipid-generated ROS and cell demise in *in vitro* and mouse
244 embryo, respectively^{71,72}. RSL3 is a GPX4 inhibitor, and an activator of ferroptosis. Hence, *GPX4*
245 overexpression reversed RSL3-mediated ferroptotic cell death while *GPX4* knockdown using short
246 hairpin RNA triggered ferroptotic cell death in malignant HRAS cells⁷². Also, iron chelators and
247 ferrostatin-1 (a lipophilic antioxidant) prevented ferroptotic cell death induced by *GPX4* deletion

248 in murine cells ^{71,73}. In addition, both *GPX4* and *GPX7* were remarkably expressed in HCC tissues
249 and this overexpression was more accentuated in grade III HCC, suggesting that *GPX4* and *GPX7*
250 overexpression play a key role in ferroptosis resistance in advanced stages of HCC ⁷⁴. Collectively,
251 these data indicate that GPX4 is cardinal for prevention of ferroptosis. In other words, GPX4 is an
252 important antioxidant component acting downstream of system xc⁻ to remove toxic phospholipid
253 hydroperoxide from membranes ⁷². Regarding the precise role of lipid peroxidation in ferroptosis,
254 a decline in the GPX4-GSH antioxidant system provokes ferroptosis ⁷⁰. Hence, inhibition of GPX4
255 by small molecule compounds such as RSL3, FIN56 or ML210, and pharmacological inhibition
256 of system xc⁻ using erastin stimulates ferroptosis (**Figs 1, 2**) ⁷². In addition, AIFM2, GCH1 and
257 DHODH-ignited intracellular antioxidant pathways play an alternative or synergistic role in
258 combating ferroptotic injury ⁷⁵. AIFM2 (a.k.a., FSP1) is an endogenous suppressor of ferroptosis
259 that functions independent of GPX4-GSH. Mechanistically, AIFM2 **myristoylation** fosters its
260 recruitment to plasma membrane. Subsequently, AIFM2, as an oxidoreductase, reduces coenzyme
261 Q10 into **ubiquinol** functioning as an antioxidant to trap lipid radicals and avert dispersion of lipid
262 peroxides ⁷⁶. Alternatively, our group revealed that AIFM2 mediated plasma membrane
263 recruitment of ESCRT III complex conferring resistance against ferroptosis through regulation of
264 membrane fission and budding and ultimate repairment of the damaged membrane independent of
265 ubiquinol (**Fig 1**) ⁷⁷. Collectively, these findings indicate that several membrane-repairing systems
266 and antioxidants co-exist to restrain ferroptosis.

267

268 **3. Downstream effectors of ferroptosis in HCC: molecular mechanisms, signaling pathways,** 269 **and therapeutic targets**

270 **3.1. NRF2**

271 NRF2, a transcription factor encoded by the *NFE2L2* gene, is tightly regulated by E3-
272 ubiquitin ligase systems including Keap1-CUL3-RBX1, bTrCP, and synoviolin ⁷⁸⁻⁸⁰. As a result
273 of endogenous/exogenous stress or interaction with binding proteins, NRF2 translocates to the
274 nucleus, where it transactivates target genes containing antioxidant response element (ARE) sites
275 ⁸¹. Intriguingly, most proteins involved in the detoxification of lipid peroxidation properties are -
276 encoded by NRF2 target genes (**Figs 1, 3, 5**) ⁸². NRF2-transactivated genes are also heavily
277 involved in the regulation of iron/heme metabolism ⁸³. For example, FTL and FTH1, as well as
278 SLC40A1, are upregulated by NRF2 (**Figs 1, 3, 5**) ^{84,85}. Moreover, *HMOX1*, *FECH*, *ABCB6*
279 (critical players in heme biosynthesis), and SLC48A1 (a transporter of heme) are all transactivated
280 by NRF2 (**Fig 5**) ⁸⁶⁻⁸⁸. Therefore, NRF2 exerts vital roles in iron/heme homeostasis, and in the
281 alleviation of lipid peroxidation and ferroptosis. Our group found that NRF2 confers protection
282 against ferroptosis in HCC cells ⁸⁹. In particular, exposure of HCC cells to ferroptosis inducers
283 (e.g., buthionine sulfoximine, sorafenib, and erastin) upregulated p62, which inactivated Keap1,
284 and averted NRF2 degradation, leading to its nuclear translocation (**Fig 3**) ⁸⁹. Subsequently,
285 nuclear NRF2 interacted with small MAF transcriptional coactivator proteins (such as MafG),
286 resulting in the upregulation of *NQO1*, *HMOX1*, *FTH1*, or *MT1G* and suppressed ferroptosis ^{89,90}.
287 Thus, knockdown of *SQSTM1* (encoding p62), *NQO1*, *HMOX1*, *FTH1* or *MT1G* sensitized HCC
288 cells to ferroptosis upon exposure to sorafenib and erastin ^{89,90}. Moreover, knockdown of *NFE2L2*
289 or pharmacological inhibition of NRF2 intensified the anticancer activity of sorafenib and erastin
290 in cell cultures and murine tumor xenograft models ⁸⁹. Thus HCC cells hijack the p62-Keap1-
291 NRF2 signaling to deter ferroptosis through upregulation of antioxidant or detoxification genes.
292 Therefore, inhibitory targeting of the p62-Keap1-NRF2 axis by genetic or pharmacological
293 inhibition along with sorafenib or erastin could be a potential therapeutic strategy to trigger the

294 ferroptotic elimination of HCC cells. It is believed that certain HCC cell lines exhibit resistance to
295 sorafenib-induced ferroptosis ⁹¹. GSTZ1, an enzyme involved in phenylalanine/tyrosine
296 catabolism, is dysregulated in various malignancies ⁹². Examination of a possible role for GSTZ1
297 in sorafenib-evoked ferroptosis revealed that sorafenib-resistant HCC cells substantially
298 downregulate the *GSTZ1* gene, leading to upregulation of *NFE2L2* and GPX4, thereby preventing
299 ferroptosis ⁹³. Furthermore, inhibition of GPX4 by RSL3 fostered sorafenib-induced ferroptosis in
300 *Gstz1*^{-/-} mice, hinting to the feasibility of a combination therapy with sorafenib and RSL3 against
301 sorafenib-resistant cells ⁹³. Moreover, forced upregulation of *GSTZ1* gene provokes ferroptosis by
302 disrupting NRF2-GPX4 signaling. Hence, genetic modulation or pharmacological maneuvers
303 affecting the GSTZ1-NRF2-GPX4 axis could be a potential therapeutic avenue in HCC therapy.
304 In addition, ABCC5, a member of ATP-binding cassette (ABC) proteins, belongs to the family of
305 ATP-dependent transporters ⁹⁴. The main biological function of ABCC5 is to extrude **xenobiotics**
306 and certain endogenous metabolites such as folic acid and cyclic GMP/AMP from cells ⁹⁵. Of
307 note, ABCC5 is responsible for sorafenib resistance and prevention of ferroptosis in HCC. In line
308 with this notion, Huang and colleagues revealed markedly elevated *ABCC5* levels in HCC cells
309 exhibiting sorafenib resistance ⁹⁶. Activation of the PI3K-AKT1-NRF2 signaling cascade was
310 suggested to facilitate *ABCC5* upregulation ⁹⁶. Moreover, *ABCC5* upregulation promoted
311 *SLC7A11* expression, downgraded lipid peroxidation and boosted intracellular GSH levels,
312 resulting in suppressed ferroptosis ⁹⁶. Of note, inhibition or downregulation of *ABCC5* promoted
313 ferroptosis *in vitro* and *in vivo* ⁹⁶. These findings denote an essential role for the PI3K-AKT1-
314 NRF2-ABCC5-SLC7A11 signaling cascade in ferroptosis resistance of HCC. Therefore,
315 pharmacological or genetic manipulation of NRF2, ABCC5, or SLC7A11 could be attempted to
316 kill HCC cells by ferroptosis. Furthermore, QSOX1 perturbs redox homeostasis through

317 suppression of NRF2/*NFE2L2* as this has been noted in human HCC ⁹⁷. QSOX1 is an enzyme
318 catalyzing thiol oxidization during protein folding, thus reducing oxygen to hydrogen peroxide ⁹⁸.
319 From a mechanistic point of view, QSOX1 mediates ubiquitin-dependent degradation of EGFR
320 and enhances its endosomal trafficking in the cytosol, resulting in suppression of *NFE2L2* ⁹⁷. Due
321 to NRF2 inhibition, QSOX1 expression facilitates sorafenib-triggered ferroptosis both *in vitro* and
322 *in vivo* ⁹⁷. Hence, activation/upregulation of *QSOX1* (genetically or pharmacologically) along with
323 sorafenib administration may induce ferroptosis in HCC and other EGFR-dependent tumors.
324 Besides, radiation therapy is the gold standard treatment in patients with unresectable HCC ⁹⁹.
325 Nonetheless, this remedy might be accompanied by radiation resistance in certain individuals. In
326 an attempt to reverse radiation resistance, Yuan and colleagues explored the potential of collectrin
327 (CLTRN) to promote radiosensitivity of HCC ¹⁰⁰. Based on the key role of CLTRN in amino acid
328 transportation through regulation of membrane distribution and solute carrier (SLC) transporters
329 ¹⁰¹, these authors postulated a relationship between CLTRN and glutathione metabolism and
330 ferroptosis ¹⁰⁰. Indeed, NRF2-RAN-DLD signaling upregulated *CLTRN* mRNA and modulated
331 glutathione metabolism in a ferroptosis-dependent manner, thereby, enhancing radiosensitivity of
332 HCC cells both *in vitro* and *in vivo* ¹⁰¹. Thus, *NRF2*-mediated upregulation of *CLTRN* may
333 predispose to ferroptosis, a notion that collides with the notion for an anti-ferroptotic role of *NRF2*.
334 Nevertheless, targeting *CLTRN*/collectrin along with radiotherapy may be an effective
335 combination therapy in the combat with HCC. Overall, these findings support a central role for
336 *NRF2* in the transcriptional regulation of anti-ferroptosis genes in HCC cells. However, the exact
337 molecular mechanisms through which the *NRF2* target genes regulate ferroptotic and non-
338 ferroptotic cell death domains remain unclear.

339

340 3.2. *YAP1/TAZ and METTL14*

341 Gao and colleagues evaluated sorafenib resistance in HCC cell lines and xenograft models
342 and found that YAP1 and TAZ transcriptional factors drastically promoted sorafenib resistance
343 via suppression of ferroptosis¹⁰². Mechanistically, YAP1/TAZ transcriptional factors upregulated
344 *SLC7A11* in a manner contingent upon TEAD1 activation¹⁰². *SLC7A11*, a crucial amino acid
345 transporter (**Fig 1**), favors intracellular glutathione production, and thereby, elicits resistance to
346 sorafenib-triggered ferroptosis¹⁰². Moreover, YAP1/TAZ transcriptional factors promoted nuclear
347 localization and activation of ATF4, which in turn, transactivated *SLC7A11*, conferring resistance
348 to ferroptosis¹⁰². This finding denotes a role for YAP1/TAZ transcriptional factors in repressed
349 ferroptosis in HCC cells, mainly through activation of the TEAD1-*SLC7A11* and the ATF4-
350 *SLC7A11* networks. Therefore, the YAP1/TAZ transcriptional factors, as well as the TEAD1-
351 *SLC7A11*/ATF4-*SLC7A11* axes may serve as targets to reverse sorafenib resistance, thus favoring
352 ferroptotic cell death in HCC cells. To corroborate the role of *SLC7A11* in ferroptosis inhibition
353 and resistance, Fan and team evaluated *METTL14* levels in HCC cells under hypoxia, revealing
354 that hypoxia suppressed *METTL14* through HIF-1 α activation, which then resulted in ferroptosis
355 inhibition through upregulation of *SLC7A11* mRNA¹⁰³. These investigators revealed that
356 *METTL14* induced N⁶-methyladenosine (refers to the methylation of adenosine at the N6-position
357 on eukaryotic mRNA) on *SLC7A11* mRNA at 5' UTR (untranslated region), predisposing it to
358 degradation dependent on the YTHDF2 signal *in vitro* and *in vivo*^{103,104}. These findings suggest
359 that hypoxia blocks ferroptosis in HCC cells through activation of the HIF-1 α -*METTL14*-
360 YTHDF2-*SLC7A11* signaling axis. Therapeutic targeting of this pathway may elicit ferroptotic
361 cell death of HCC cells, contrasting with the finding that HIF-1 α plays an active role in promoting

362 ferroptosis in renal cancers ^{105,106}. More studies are needed to determine the cell-specific role of
363 hypoxia signaling in shaping ferroptosis sensitivity.

364

365 **3.3. Circular and long non-coding RNAs**

366 Single-stranded RNAs form a covalently bound loop, commonly referred to as circular
367 RNAs (circRNAs) ¹⁰⁷. Such non-coding RNAs participate in tumorigenesis and may also affect
368 ferroptosis ¹⁰⁷. Circular-interleukin-4 receptor (*circIL4R*) is highly expressed in HCC cells ¹⁰⁸. Xu
369 and colleagues studied the role of *circIL4R* in HCC progression and ferroptosis and revealed that
370 *circIL4R* conferred protection against ferroptosis ¹⁰⁹. Knockdown of *circIL4R* elicited ferroptosis
371 and thereby impeded oncogenesis ¹⁰⁹. Mechanistically, *circIL4R* directly suppressed *miR-541-3p*,
372 leading to increased expression of *GPX4* mRNA (a direct target of *miR-541-3p*) and disabled
373 ferroptosis ¹⁰⁹. These data insinuate an oncogenic role for *circIL4R* that is largely imputed to
374 ferroptosis inhibition through activation of *circIL4R-miR-541-3p-GPX4* axis in HCC. Hence,
375 silencing *circIL4R* or pharmacological blockade of the *circIL4R-miR-541-3p-GPX4* axis could
376 facilitate ferroptosis in HCC. In a recent study, Lyu and coworkers reported that *circ0097009* was
377 remarkably increased, leading to enhanced invasion and proliferation of HCC cells in murine
378 xenograft models. However, these effects were reversed by *circ0097009* knockdown ¹¹⁰. Further
379 analysis disclosed that *SLC7A11* and *circ0097009* directly and concurrently bound to microRNA
380 (*miR*)-1261 ¹¹⁰. Mechanistically, *circ0097009* directly binds and blocks *miR-1261*, thereby
381 inducing *SLC7A11* upregulation and ferroptosis inhibition, leading to proliferation and invasion
382 of HCC cells ¹¹⁰. These data suggest that HCC cells activate the *circ0097009-miR-1261-SLC7A11*
383 axis to revert ferroptosis, hence revealing yet another potential target for expediting ferroptosis in
384 HCC cells. Moreover, examining causal correlations among *circ0013731*, *miR-877-3p*, and

385 *SLC7A11* showed that *circ0013731* was significantly upregulated in an E2F1-dependent fashion
386 ¹¹¹. *circ0013731* boosted cell growth due to suppressed ferroptosis in HCC cells (QGY-7703,
387 SMMC-7721) and xenograft models ¹¹¹. Mechanistically, *circ0013731* upregulates *SLC7A11* by
388 direct binding to and sponging *miR-877-3p* ¹¹¹. These findings suggest that HCC cells adapt the
389 E2F1-*circ0013731-miR-877-3p-SLC7A11* axis to prevent ferroptosis. Qi and colleagues reported
390 that erastin induced upregulation of the long non-coding RNA (lncRNA) *GABPB1-AS1*, which
391 favors ferroptosis induction ¹¹². Mechanistically, *GABPB1-AS1*-induced downregulation of
392 *GABPB1* resulted in *PRDX5* downregulation, thus, reducing the cellular antioxidant potential and
393 inducing ferroptosis in HepG2 cells ¹¹². Hence, bolstering the *GABPB1-AS1-GABPB1-PRDX5*
394 axis by genetic or pharmacological means might be a potential approach to sensitize HCC to
395 ferroptosis. Taken together, current research might inspire RNA-based ferroptosis therapeutics in
396 HCC pending additional mechanistic and translational exploration.

397

398 **3.4. SPARC, ATP-binding cassette, and PERK**

399 SPARC participates in interactions and communications between cells and extracellular
400 matrix (ECM), to govern cellular functions including differentiation, proliferation, and adhesion
401 ¹¹³. Nonetheless, its role in HCC progression and sorafenib sensitivity remains largely elusive. Wei
402 and associates reported that SPARC enhanced sorafenib cytotoxicity in HCC cell lines (e.g.,
403 HepG2 and Hep3B) ¹¹⁴. Thus, abrogation of SPARC expression diminished sorafenib-mediated
404 killing of these cell lines ¹¹⁴. Conversely, SPARC overexpression promoted ferroptosis through
405 excessive oxidative stress, thus, enhancing sorafenib cytotoxicity ¹¹⁴. These findings indicate that
406 SPARC is a potential target to further sensitize HCC cells to sorafenib-induced ferroptosis. ATP-
407 binding cassette (ABC) transporters belong to a family of membrane proteins, the function of

408 which remains largely elusive in HCC ¹¹⁵. Bioinformatic analyses by Zhang and associates
409 uncovered that ABCA8 and ABCA9 are markedly downregulated, while ABCA6 is upregulated
410 in HCC cells ¹¹⁵. Further analysis showed that ABCB6 participates in the regulation of ferroptosis
411 ¹¹⁵. This finding spurs further research to explore the mechanisms underlying ABCB6-regulated
412 ferroptosis and its role in oncogenesis. In particular, identification of ABC substrates may provide
413 a new framework for understanding the metabolic basis of ferroptosis. PERK is an endoplasmic
414 reticulum (ER) stress sensor and ER transmembrane protein ¹¹⁶. Of note, in HCC cells irradiation
415 triggered ER stress with consequent PERK signaling, which in turn, induced expression of *ATF4*
416 and *DRAM1*. Moreover, PERK-induced expression of *p53* stimulated apoptosis and ferroptosis via
417 downregulation of *SLC7A11*, hence sensitizing HepG2 cells to irradiation ¹¹⁷. Combination of
418 carbon ion irradiation with sorafenib triggered a mixed-type of apoptotic and ferroptotic cell death
419 ¹¹⁷. Thus, induction of ER stress, in particular activation of the PERK-p53 pathway, may promote
420 ferroptosis in HCC cells.

421

422 **3.5. S1R, ceruloplasmin, and retinoblastoma**

423 The ligand-operated sigma-1 receptor (S1R) is a multi-functional protein residing in
424 endoplasmic reticulum (ER) membranes ¹¹⁸. S1R is assumed to regulate oxidative stress in an
425 NRF2-dependent manner ¹¹⁹, and thereby governs the regulation of ferroptosis. Bai and coworkers
426 reported that sorafenib triggered-nucleus translocation of S1Rs, while inhibition of ferroptosis with
427 ferrostatin-1 reversed this translocation in HCC cells, suggesting a role for enhanced S1R levels
428 in ferroptosis inhibition ¹²⁰. Abrogation of S1R promoted sorafenib-induced ferroptosis due to
429 repression of *GPX4*, elevation of lipid peroxidation, and iron metabolism in HCC *in vitro* and *in*
430 *vivo* ¹²⁰. Interestingly, NRF2 inhibition promoted S1R expression and conferred protection against

431 ferroptosis ¹²⁰. Overall, these findings suggest that in response to sorafenib, HCC cells upregulate
432 S1R to resist ferroptosis and maintain survival. Meanwhile, inhibition of NRF2, which predisposes
433 to ferroptosis, leads to S1R upregulation and subsequent inhibition of ferroptosis, suggesting that
434 HCC cells would employ this “smart system” (NRF2-S1R) to resist ferroptosis under conditions
435 of NRF2 inhibition. A more profound exploration of the role of S1R in ferroptosis is warranted.
436 On the other hand, the glycoprotein ceruloplasmin plays a cardinal role in iron homeostasis ¹²¹. In
437 HCC cells, ceruloplasmin blocks ferroptosis by modulating iron homeostasis ¹²². Ceruloplasmin
438 depletion accelerates ferroptosis by increasing intracellular accumulation of Fe²⁺ and lipid-
439 associated ROS in HCC cells under treatment with RSL3 or erastin ¹²². Conversely, overexpression
440 of *CP* prevents ferroptosis ¹²². These data indicate that upregulation of *CP*/ceruloplasmin fends off
441 ferroptosis via reduction of intracellular Fe²⁺ ¹²². Since ceruloplasmin plays an important role in
442 storing and carrying copper, it remains to be seen whether copper affects ferroptosis resistance as
443 well. Besides, retinoblastoma denotes a tumor suppressor protein, which is inactivated in several
444 malignancies ¹²³. Retinoblastoma also plays a crucial role in liver carcinogenesis, and depletion of
445 retinoblastoma protein promotes sorafenib-induced ferroptosis in human HCC cells both in vitro
446 and in xenograft settings ¹²⁴. Hence, as a potential combination therapy, suppression of
447 retinoblastoma along with sorafenib administration might abrogate HCC progression.

448

449 ***3.6. G6PD, C1SD2 and C1SD1, and NUPR1***

450 G6PD is an enzyme in the pentose phosphate pathway, a sequence of biochemical reactions
451 that convert glucose into ribose-5-phosphate (**Fig 5**) ¹²⁵. G6PD is often upregulated in HCC. Cao
452 and colleagues reported that G6PD buttressed invasion, migration, and proliferation of HCC *in*
453 *vivo* by inhibiting ferroptosis through downregulation of POR, a type of cytochrome P450

454 oxidoreductase ¹²⁶. Indeed, *G6PD* knockdown resulted in the abatement of tumor weight and
455 volume ¹²⁶. Hence, the G6PD-POR axis might constitute yet another potential maneuverable target
456 to ignite ferroptosis in HCC, pending confirmation and further mechanistic exploration of this
457 pathway. In addition, the protein C1SD2 is localized at the ER and mitochondrial outer membrane
458 (MOM) ¹²⁷. C1SD2 serves as a key regulator for cellular processes including intracellular Ca²⁺
459 homeostasis, mitochondrial function, and ER integrity (**Fig 3**) ¹²⁷. It has been evinced that C1SD2
460 is associated with HCC progression. *Li* and team demonstrated that C1SD2 was overly upregulated
461 in HCC cells, contributing to disease progression and sorafenib resistance ¹²⁸. Thus, *C1SD2*
462 knockdown reversed sorafenib resistance, reduced viability, and provoked ferroptosis in HCC cells
463 ¹²⁸. Mechanistically, *C1SD2* knockdown triggered excessive Beclin1-dependent autophagy and
464 ferritinophagy, which spurred ferroptosis in sorafenib-resistant HCC cells ¹²⁸. Moreover, genetic
465 inhibition of C1SD1 favored iron-mediated intramitochondrial lipid peroxidation, contributing to
466 erastin-induced ferroptosis in human HCC cells ¹²⁹. Hence both C1SD1 and C1SD2 may affect
467 ferroptosis sensitivity in HCC cells. Besides, NUPR1 is a small chromatin-binding protein that
468 activates and regulates gene expression in response to various types of cellular stress. NUPR1 was
469 originally identified as a ferroptosis suppressor in pancreatic cancer cells, promoting iron export
470 by activating LCN2 expression ¹³⁰. Subsequent studies confirmed a role for NUPR1 in suppressing
471 ferroptosis by reducing mitochondrial oxidative damage in HCC cells ¹³¹. Consequently, the
472 NUPR1 inhibitor ZZW-115 can increase the sensitivity of pancreatic cancer cells and HCC cells
473 to ferroptosis ^{130,131}. These findings establish a nuclear factor pathway to limit oxidative damage
474 during ferroptosis.

475

476 **3.7. Other effectors**

477 Examination of miRNA-related epigenetic modifications and their clinical relevance
478 revealed that miRNA (*miR*)-23a-3p is predominantly overexpressed in HCC, in association with
479 sorafenib resistance, HCC relapse, poor survival, as well as suppression of ferroptosis, lipid
480 peroxidation, and iron accumulation ¹³². Mechanistically, activation of ETS1 contributed to *miR*-
481 23a-3p upregulation, which in turn, targeted 3'-UTR of *ACSL4* mRNA, resulting in ferroptosis
482 suppression ¹³². Notably, CRISPR/Cas9-mediated *miR*-23a-3p knockout boosted the sorafenib
483 response in HCC cells and orthotopic HCC xenografts ¹³². Ferroptosis is linked to selective
484 autophagy categories including chaperone-mediated autophagy, clockophagy, lipophagy, and
485 ferritinophagy (**Fig 6**). However, selective autophagy of ER fragments, which is referred to as
486 reticulophagy or ER-phagy ¹³³, is less explored regarding its possible role in ferroptosis. In a recent
487 study, Liu and coworkers described that sorafenib induced FAM134B-dependent reticulophagy,
488 conferring resistance to ferroptosis in HCC *in vitro* and *in vivo* ¹³⁴. PABPC1 was found to interact
489 with *FAM134B* mRNA, thereby upregulating its translation ¹³⁴. *FAM134B* knockdown suppressed
490 reticulophagy and overtly promoted ferroptosis sensitivity ¹³⁴. These data suggest that activation
491 of PABPC1-FAM134B-reticulophagy in response to sorafenib treatment may serve as an adaptive
492 mechanism of HCC cells to prevent ferroptosis. Therefore, targeting this pathway might reverse
493 sorafenib resistance. Also, HCC radioresistance is coupled to reduced *COMMD10* expression post-
494 irradiation, leading to enhanced intracellular copper levels, thus suppressing ferroptosis ¹³⁵.
495 Mechanistically, cytosolic copper accumulation rescued HIF1- α from ubiquitin degradation,
496 favoring its translocation to the nucleus and transactivation of *CP* (encoding ceruloplasmin) and
497 *SLC7A11*, resulting in ferroptosis suppression. Conversely, *COMMD10* overexpression triggered
498 radiosensitivity via induction of ferroptosis both *in vitro* and *in vivo* ¹³⁵. Moreover, a recent
499 CRISPR/Cas9 screening uncovered a role for PSTK in reducing therapeutic sensitivity of HCC

500 cells ¹³⁶. PSTK suppressed sorafenib-induced ferroptosis, while its depletion inactivated GPX4
501 and GSH metabolism as well as the synthesis of cysteine and selenocysteine, hence sensitizing
502 cells to sorafenib *in vitro* and *in vivo* ¹³⁶. Similarly, the PSTK inhibitor punicalin reverted sorafenib
503 resistance of HCC ¹³⁶, supporting future translational studies of PSTK as a potential target for
504 ferroptosis sensitization.

505

506 **4. Potential ferroptosis inducers in HCC therapy**

507 As indicated by aforementioned studies, ferroptosis induction suppresses the growth and
508 progression of HCC and promotes chemotherapeutic sensitization. Hence, small molecules that
509 upregulate ferroptosis might be useful for HCC therapy. Here we provide a short list of natural and
510 synthetic agents that might be considered as the lead compounds for the future development of
511 ferroptosis inducers. Artesunate, which is primarily employed for malaria treatment, is clinically
512 well-tolerated. ¹³⁷. Li and colleagues reported that artesunate synergistically boosted the anticancer
513 effects of sorafenib through enhancement of lipid peroxidation and ferroptosis in HCC cells (SNU-
514 182, SNU-449, Huh7) and murine xenograft models (Balb/c nude) ¹³⁸. Moreover, combination
515 treatment of artesunate and sorafenib elevated oxidative stress and ferritinophagy, thereby,
516 upregulating ferroptosis in Huh7 cells ¹³⁸. In this system, artesunate induced activation of
517 lysosomal **cathepsin B/L**, lipid peroxidation, and ferritinophagy ¹³⁸. Pending additional preclinical
518 confirmation, these findings suggest that artesunate could be utilized in combination with
519 sorafenib. Also, the anticancer agent atractylodin ¹³⁹ inhibits invasion, migration, and proliferation
520 of HCC cells including Hcm and Huh7 ¹⁴⁰. Moreover, atractylodin elevated ROS levels,
521 downregulated *GPX4* and *FTL*, and upregulated *TFRC* and *ACSL4*, thus contributing to ferroptosis
522 ¹⁴⁰. Formosanin C is a natural saponin used in Chinese herbal medicine to alleviate inflammation

523 ¹⁴¹. As described earlier, a rise in iron pool in response to ferritinophagy promotes ferroptosis (**Figs**
524 **1, 4**) ⁴⁵. Su and colleagues found that formosanin C inhibited HCC cell growth (Hep3B, HepG2)
525 and that administration of the ferroptosis inhibitor ferrostatin-1 reversed this effect ¹⁴².
526 Mechanistically, formosanin C induced NCOA4-dependent ferritinophagy, leading to Fe²⁺
527 overload and ferroptosis ¹⁴². Moreover, formosanin C triggered ferroptosis and autophagic flux in
528 HepG2 cells while enhancing *NCOA4* and reducing *FTH1* mRNA levels¹⁴³. Moreover, formosanin
529 C-induced ferritinophagy triggers ferroptosis in HA59T and HA22T cells ¹⁴³, confirming its
530 potential therapeutic utility. In addition, haloperidol, a drug used for treatment of schizophrenia
531 ¹⁴⁴, may substantially potentiate sorafenib- or erastin-induced ferroptosis in HCC ¹⁴⁵. At a
532 relatively low dose, haloperidol effectively triggered intracellular Fe²⁺ overload and lipid
533 peroxidation ¹⁴⁵. Therefore, haloperidol is a potential ferroptosis inducer that should be further
534 investigated at the preclinical level. Heteronemin, derived from the marine sponge
535 *Hippospongia* sp., has anticancer properties against myeloid leukemia, prostate cancer, and
536 cholangiocarcinoma ¹⁴⁶. Heteronemin also blocks proliferation of HA59T and HA22T cell lines
537 through initiation of caspase-dependent apoptosis ¹⁶. Moreover, heteronemin induces ROS
538 production and GPX4 downregulation, resulting in ferroptotic cell death ¹⁶. Hence, ferroptosis
539 inhibition attenuated heteronemin-mediated cell death ¹⁶. **Table. 1** lists newly identified ferroptosis
540 inducers in the HCC therapy.

541

542 **5. Targeting NRF2 in HCC therapy**

543 Ample evidence has indicated that NRF2 inhibition remarkably boosts anticancer effects
544 of ferroptosis inducers in HCC and other cancers. For instance, trigonelline, a natural alkaloid
545 abundantly found in plants (e.g., oats, hemp seed, garden peas, and coffee beans), promotes

546 ferroptosis under sorafenib treatment due to NRF2 inhibition in HCC cells and xenograft models
547 ⁸⁹. Although trigonelline cannot be considered as a specific NRF2 inhibitor, targeting NRF2 seems
548 to be promising for ferroptotic elimination of HCC cells and more specific NRF2 inhibitors should
549 be developed. Disulfiram, which is in clinical use for alcoholism therapy, remarkably inhibited
550 angiogenesis, invasion, and migration in HCC cell lines, as it induced mitochondrial impairment,
551 lipid peroxidation, and intracellular iron overload, resulting in ferroptotic cell death ¹⁴⁷. The
552 elevation of *NRF2* expression induced resistance to disulfiram, while *NRF2* inhibition by RNA
553 interference remarkably accelerated lipid peroxidation and facilitated disulfiram-triggered
554 ferroptosis ¹⁴⁷, suggesting the benefit of NRF2 inhibition. The use of high-throughput screening
555 (HTS) in association with cell-based assays has made it possible to discover potential anticancer
556 drugs and to identify new applications for already FDA-approved drugs ¹⁴⁸. Using this technique,
557 AEM1 has been revealed as a small molecule that interferes with transcriptional activity of NRF2,
558 resulting in growth suppression and enhanced chemosensitivity of A549 cancer cells both *in vitro*
559 and *in vivo* ¹⁴⁹. Moreover, Singh and colleagues screened 400,000 small molecules and found that
560 the compound ML385 targets NRF2 with high selectivity and specificity. Indeed, combination of
561 ML385 with carboplatin exhibited remarkable anticancer efficacy in preclinical models of NRF2-
562 addicted non-small cell lung cancer (NSLC) ¹⁵⁰. Recently, Matthews and coworkers screened
563 several RNA interference and chemical libraries to identify actin disturbing agents, STAT3
564 inhibitors, and cardiac glycosides as NRF2 inhibitors that synergized with anticancer drugs in
565 killing A549 NSLC cells ¹⁵¹. These findings broaden the list of potential compounds with NRF2-
566 inhibitory effects, and pave the way for characterization and identification of new NRF2 inhibitors
567 with more specificity and sensitivity. **Fig 6** briefly summarizes potential natural and synthetic

568 therapeutics targeting NRF2 in HCC. **BOX 1** also represent the utility of nanotechnology in
569 ferroptosis induction.

570

571 **6. Ferroptosis and the efficacy of HCC immunotherapy**

572 In a clinical study, a risk assessment model was established based on differential expression
573 of ferroptosis-related genes to predict survival and prognosis of immunotherapy efficacy in
574 patients with HCC¹⁵². Based on sample analyses, patients were categorized into two groups; low-
575 ferrscore group (harboring less differentially expressed ferroptosis-related genes) and high-
576 ferrscore group (harboring high differentially expressed ferroptosis-related genes). As a result,
577 high-ferrscore group exhibited a remarkably lower survival rate than the other group. Besides,
578 expression of *PD-1* and *PD-L1* was markedly greater in the high-ferrscore than low-ferrscore
579 group. Thus, immunotherapy efficacy was unsatisfactory in the high-ferrscore group following
580 treatment with PD-1 and CTLA4 inhibitors (immune checkpoint inhibitors)¹⁵². Collectively, these
581 clinical findings indicate that highly differentiated expression of ferroptotic genes in HCC cells
582 renders resistance to immunotherapy. Also, in an independent study, **transcriptome** and
583 **methyloome** analyses of ferroptosis-related genes were carried out in 374 patients with HCC to
584 determine ferroptosis-associated HCC subtypes¹⁵³. Subsequently, ferroptosis-associated HCC
585 subtypes were explored for their link with tumour immune microenvironment. As a result, two
586 distinct phenotypes including ferroptosis-L and ferroptosis-H were recognized based on
587 methylation and expression of ferroptotic genes in HCC patients. Ferroptosis-H group had a lower
588 survival rate and different microenvironmental immune status¹⁵³. Besides, 15 ferroptosis-
589 associated genes were identified as a prognostic and risk stratification model for accurate decision-
590 making in clinical treatment and immunotherapy of HCC¹⁵³. In another setting, HCC patients

591 were analyzed using bioinformatic techniques to reveal a ferroptosis-associated
592 diagnostic/prognostic model useful for prediction of immune activity ¹⁵⁴. Hence, a prognostic
593 model was developed based on *SLCIA5* and *SLC7A11* mRNAs and 8 ferroptosis-associated long
594 non-coding RNAs (lncRNAs), which were proven to function better than pathological
595 characteristics ¹⁵⁴. Collectively, this finding represented a ferroptosis-associated model based on
596 mRNA and lncRNA molecules for prognosis and prediction of immune activity, thereby, offering
597 a guideline for successful immunotherapies.

598

599 **7. Concluding remarks and future perspectives**

600 Within the last 5 years, research on ferroptosis in HCC has advanced considerably, and
601 many small-molecule compounds or drugs were developed to induce ferroptosis to suppress tumor
602 growth. Despite these prominent advances in the field, ferroptosis resistance remains a challenge
603 for translational application. Following treatment with ferroptosis activators, the surviving fraction
604 of HCC cells can undergo a process of "redox reset" with a stronger antioxidant system. Notably,
605 transcriptional factor NRF2 orchestrates the stress-induced activation of numerous cytoprotective
606 genes to suppress ferroptosis. Understanding the regulation of NRF2 activity and its downstream
607 signaling may contribute to the pharmacological subversion of this "redox reset" and hence to the
608 avoidance of ferroptosis resistance. Based on the available literature, we speculate that ignition of
609 a single pathway of ferroptosis is unlikely to achieve a durable ferroptotic response that affects the
610 vast majority of HCC cells within a tumor. Instead, combination efforts should be sought in which
611 several pathways are concurrently turned on. A better understanding of tumor microenvironment,
612 especially the communication between immune cells and cancer cells, may provide new insights
613 into the immunological signature of ferroptosis, which can aid in the design of new drugs and

614 combination therapies ¹⁵⁵. Indeed, in an ideal scenario, induction of ferroptosis should stimulate or
615 at least be compatible with an anticancer immune response that keeps residual HCC cells. Last but
616 not least, the search for specific, user-friendly biomarkers to predict ferroptosis responses will be
617 important for design of targeted therapies ¹⁵⁶. Thus, identification of drivers and genetic or
618 metabolic vulnerabilities of ferroptosis that affect malignant but not normal cells, may gear
619 customized interventions in individual patients ¹⁵⁷.

620

621 **Declarations**

622 **Ethical Approval and Consent to participate**

623 Not applicable.

624

625 **Consent for publication**

626 Not applicable.

627

628 **Availability of supporting data**

629 Not applicable.

630

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644 AA has written the initial draft of the manuscript and DT, GK, and JR have contributed to
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660

661 **Table. 1.** Recently identified ferroptosis inducers for HCC therapy

Compounds	Mechanisms of Action	Refs
DSF (disulfiram)	Remarkably inhibits angiogenesis, invasion, and migration, induces mitochondrial impairment, lipid peroxidation, and intracellular iron overload, resulting in ferroptotic cell death	¹⁴⁷
Heteronemin (natural marine Terpenoid derived from <i>Hippospongia sp</i>)	Inhibits HCC cell lines (HA22T and HA59T) proliferation by inducing apoptosis and ferroptosis via inhibition of <i>GPX4</i> and <i>ERK</i> expression	¹⁶
Necrostatin-1 (TNF- α inhibitor)	Blocks ferroptosis due to its antioxidant capacity independent of RIPK1 inhibition in HCC cells (SK-HEP-1 and Huh7)	¹⁵⁸
Solasonine (a glycoalkaloid)	Remarkably suppresses proliferation, invasion, and migration in HepRG and HepG2 cells and in mouse xenograft models via ferroptosis and GPX4 suppression	¹⁵⁹
Triapine (lactose-decorated nanomicelles loaded with triapine and photosensitizer Ce6)	Activates fenton reaction, ROS generation, and ferroptosis both <i>in vitro</i> and in HCC orthotopic mice	¹⁶⁰

662

663 • **BOX 1. Nanotechnology for ferroptosis induction in HCC therapy**

664 Owing to the peculiar physicochemical characteristics of nanomaterials, nanotechnology
 665 might open the avenue to future antineoplastic approaches. Although research on
 666 nanotechnology-based cancer treatment constitutes an expanding area, so far no clinical

667 translation of these approaches are at sight ¹⁶¹. Given that some characteristics (such as pH,
668 oxygen tension and bloodflow) of the tumor microenvironment (TME) are distinct from
669 those of healthy tissues, nanomaterials can be designed to specifically reach tumor cells
670 without affecting normal cells ¹⁶²⁻¹⁶⁴. In recent years, nanomedicine has participated to the
671 development of ferroptosis inducers ^{165,166}. Construction of nano ferroptosis inducers
672 constitutes conjugation or incorporation of small ferroptosis inducers on or into
673 nanocarriers that are supposed to boost the biocompatibility, solubility, and tumor
674 localization of the cytotoxic drugs ¹⁶⁷. Alternatively nanomaterials can be developed with
675 the scope to generate hybrid molecules that combine ferroptosis induction with malignant
676 cell targeting ^{167,168}. For the treatment of HCC, Sorafenib is the only FDA-approved
677 chemotherapeutic and ferroptosis inducer. Nonetheless, sorafenib administration is
678 associated with many pitfalls including toxic side effects, poor bioavailability and water
679 solubility ¹⁶⁹. In a recent study, Yue and colleagues incorporated sorafenib into pH-
680 sensitive HMPB (hollow mesoporous Prussian blue) nanoparticles, thereby generating a
681 sorafenib nanocarrier with facilitated drug release ¹⁷⁰. They also conjugated pH-responsive
682 chitosan to the HMPB surface to boost the biocompatibility of sorafenib ¹⁷⁰. The resulting
683 tripartite HMPB-sorafenib-chitosan nanocomposite exhibited enhanced specificity for
684 HCC cells and less toxicity to normal tissues due to improved permeability and retention
685 *in vitro* and *in vivo* ¹⁷⁰. Mechanistically, nanocomposite ignited excessive ROS generation
686 and ferroptosis, resulting in HCC shrinkage in mice ¹⁷⁰. In another study, MMSNs
687 (manganese-silica nanoparticles) induced ferroptosis after uptake of the particles into HCC
688 cells and their degradation, which resulted in rapid scavenging of intracellular GSH ¹⁷¹.
689 Moreover, loading MMSNs with sorafenib facilitated specific targeting of the HCC TME

690 ¹⁷¹. Thus, MMSNs can act as a ferroptosis inducer, as well as a sorafenib carrier.
691 Altogether, nanomaterials may be advantageous for the development of novel ferroptotic
692 inducers with favorable galenic properties. Clinical trails are needed to validate their
693 efcacies.

694

695 • **Figure Legends**

696 **Fig. 1. Iron metabolism, overload, and ferroptosis**

697 The antiporter SLC7A11 governs GSH production, which is utilized by GPX4 to neutralize
698 lipid peroxidation. Extracellular Fe³⁺ binds to transferrin, which in turn, binds to TFR1 for
699 endocytosis. In endosomes, Fe³⁺ is converted into Fe²⁺ prior to release into the cytosol via
700 SLC11A2, leading to Fe²⁺ overload, ROS production, lipid peroxidation, ultimately,
701 ferroptosis. Lipid peroxidation also enjoys participation of lipid metabolism enzymes such
702 as PLA2G2A, ALOX15, LPCAT3, and ACSL4. Intracellular Fe²⁺ is also exported through
703 SLC40A1 or stored as Fe³⁺ within ferritin. Mitochondrial Fe²⁺ overload, a direct result of
704 cytosolic Fe²⁺ overload, triggers ROS generation, thus, promoting lipid peroxidation and
705 ferroptosis. Moreover, ferritinophagy is another process leading to Fe²⁺ overload. The
706 transcriptional factor NRF2 upregulates many genes encoding proteins with a role in ROS
707 and lipid peroxidation suppression. Under ER stress, activation of the ATF4 transcriptional
708 factor upregulates HSPA5 chaperone, which suppresses lipid peroxidation, likely through
709 promoting GSH folding in the ER.

710

711 **Fig. 2. Ferroptosis mechanisms and regulations**

712 The anti-transporter systems of SLC7A11 and SLC3A2 are primarily responsible for
713 cystine uptake in oxidative extracellular environment. With the reductive extracellular
714 environment, SLC7A10 mediates direct uptake of cysteine into the cytosol, leading to
715 activation of GPX4. Moreover, intracellular cysteine levels can be replenished by
716 conversion of methionine amino acid into cysteine through transsulfurylation mechanism.
717 The FAT/FATP system mediates uptake of arachidonic acid and adrenoyltic acid, which
718 are key substrates for initiation of lipid peroxidation. Finally, various compounds can target
719 GPX4, Fe²⁺ overload, and SLC7A11-SLC3A2 system thereby, manipulating ferroptotic
720 fate of the cell.

721

722 **Fig. 3. Signaling pathways and regulation of ferroptosis**

723 Cell signaling pathways play a role in regulation of ferroptosis by modulating the activity
724 and transcription of SLC7A11, labile iron pool, and lipid peroxidation. ER stress and
725 activation of PERK signaling also affect GPX4 and therefore, lipid peroxidation.

726

727 **Fig. 4. Autophagy and ferroptosis**

728 Selective types of autophagy including lipophagy, ferritinophagy, mitophagy, chaperone-
729 mediated autophagy, and clockophagy can promote ferroptosis via increased lipid
730 peroxidation, ROS generation, and Fe²⁺ overload.

731

732 **Fig. 5. NRF2-keap1 pathway dictates degradation or nuclear translocation of NRF2**

733 Under normal conditions, Keap1 in association with CUL3 induce proteasomal degradation
734 of NRF2. However, under stress conditions, Keap1 cysteine residues are altered, leading to
735 NRF2 dissociation from Keap1. As a result, NRF2 translocates to the nucleus and
736 transactivates genes involved in suppression of lipid peroxidation and ferroptosis.

737

738 **Fig. 6. Potential compounds targeting NRF2 at different stages of HCC progression.**

739 Given the role of NRF2 in preventing HCC cells from ferroptotic cell death, potential
740 natural/pharmacological therapeutics inhibiting transcription, translation, nuclear
741 translocation, and DNA binding of NRF2, or promoting its degradation could be promising
742 tools in the neutralization of NRF2 oncogenic activities.

743

744 • **Glossary**

745 **Autophagy:** An evolutionarily conserved process in eukaryote cells mediating engulfment
746 of damaged organelles or cellular components within transitory organelles, so-called,
747 autophagosomes, which subsequently, fuse with lysosomes for ultimate degradation of the
748 engulfed cargo.

749 **Cathepsin B/L:** Cathepsin B/L are lysosomal cysteine proteases playing a role in cellular
750 functions including intracellular proteolysis.

751 **Chronic hepatitis:** Refers to liver inflammation and impairment inflicted by hepatitis B
752 and C viruses and drug toxicity.

753 **Ferrireductases:** A group of enzymes that mediate the reduction of Fe^{3+} to Fe^{2+} .

754 **Hemochromatosis:** A disorder caused by accumulation and build-up of extra iron in the
755 body.

756 **Methylome:** A technique analyzing distribution of 5-methylcytosine in the entire genome.

757 **Myristoylation:** A lipid modification mechanism in which a fatty acid known as myristic
758 acid binds to the N-terminal domain of a protein.

759 **Non-alcoholic steatohepatitis (NASH):** Refers to liver inflammation and impairment
760 induced by fat accumulation in the liver.

761 **Saponin:** Natural glycosides, which constitute sugars like apiose, arabinose, galactose, and
762 glucose, are found abundantly in plants.

763 **Transcriptome:** Refers to a thorough range/record of expressed mRNAs within an
764 organism.

765 **Xenobiotics:** Refers to chemical compounds that are naturally produced or exists within
766 an organism.

767

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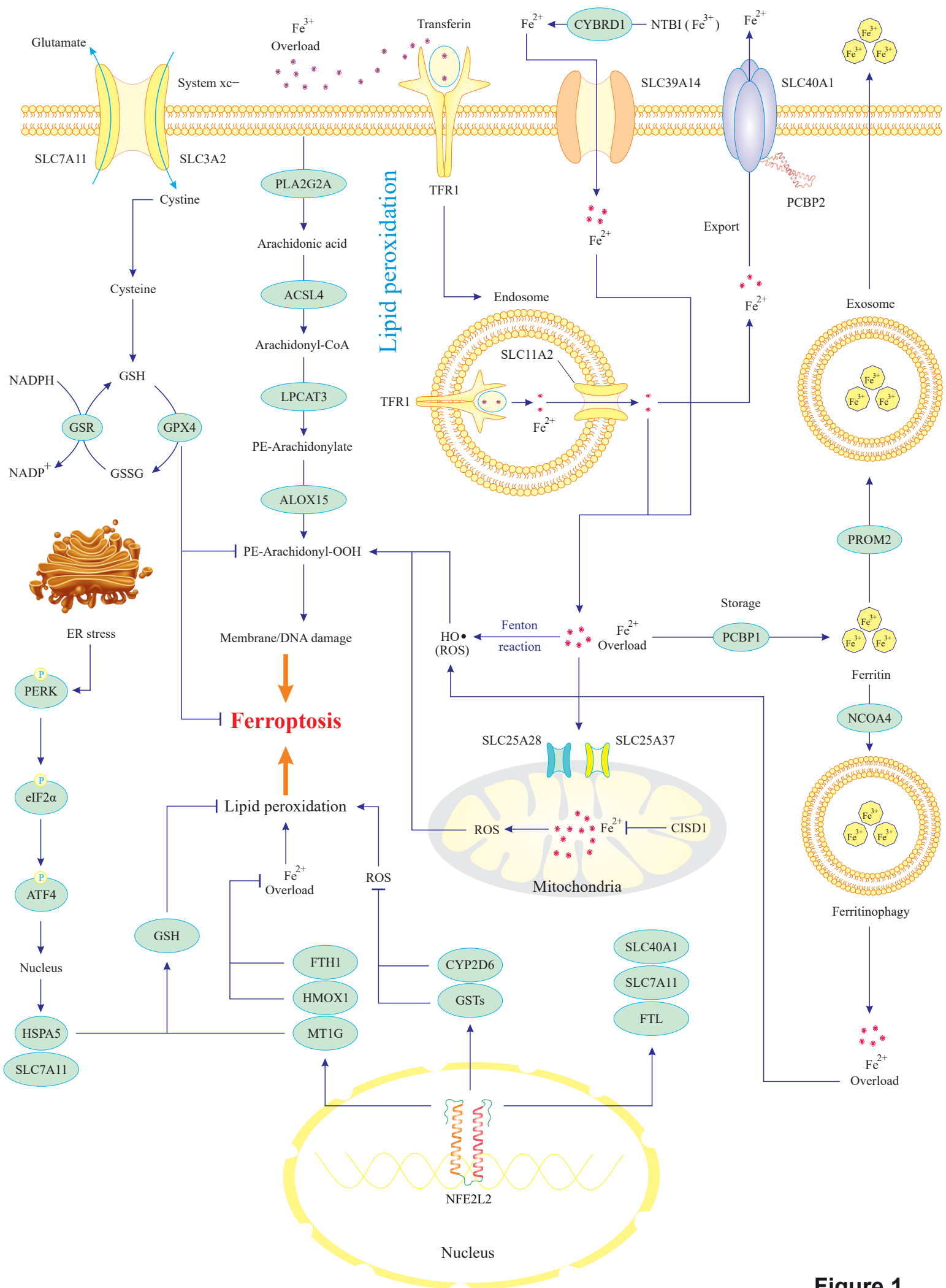


Figure 1

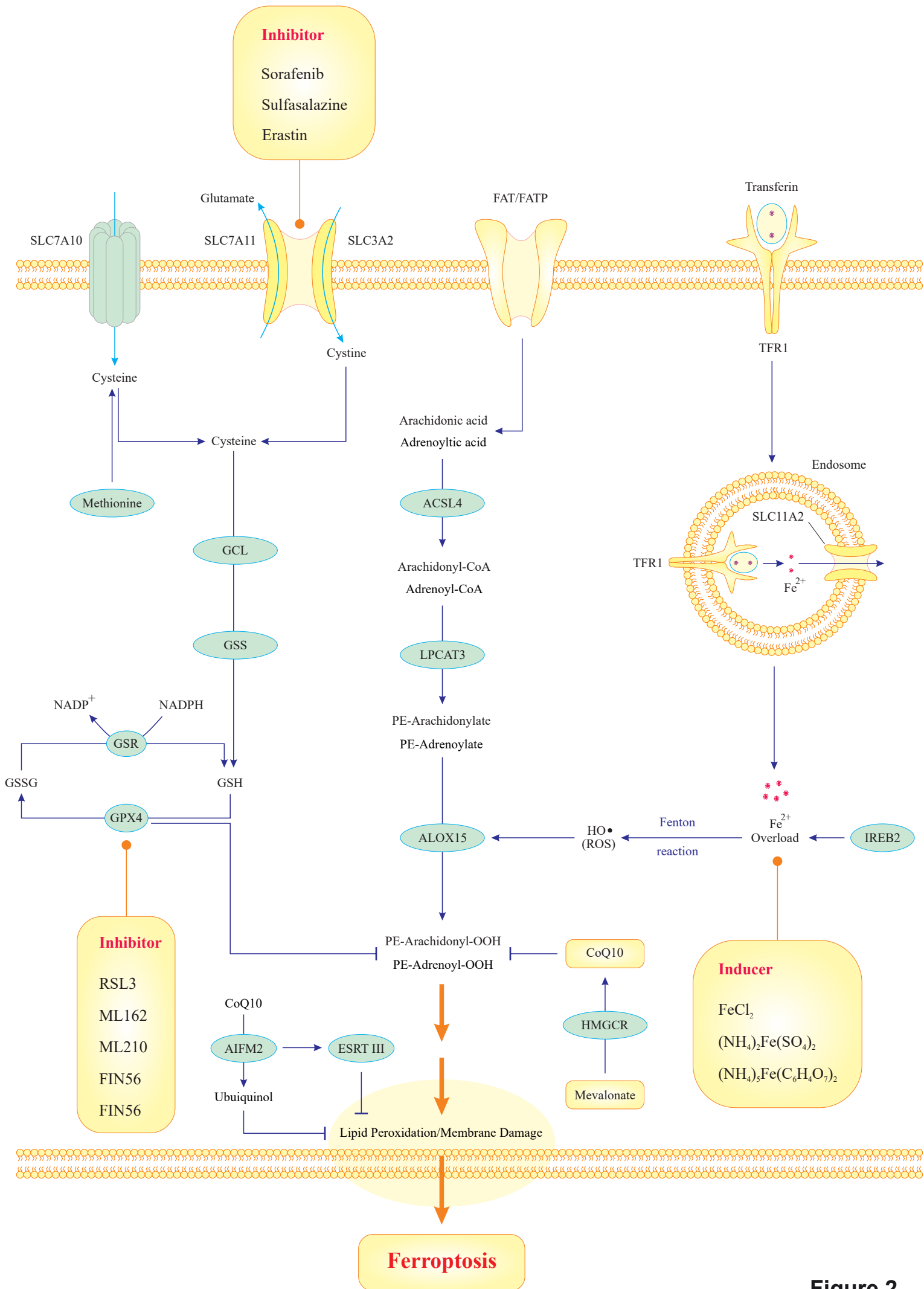


Figure 2

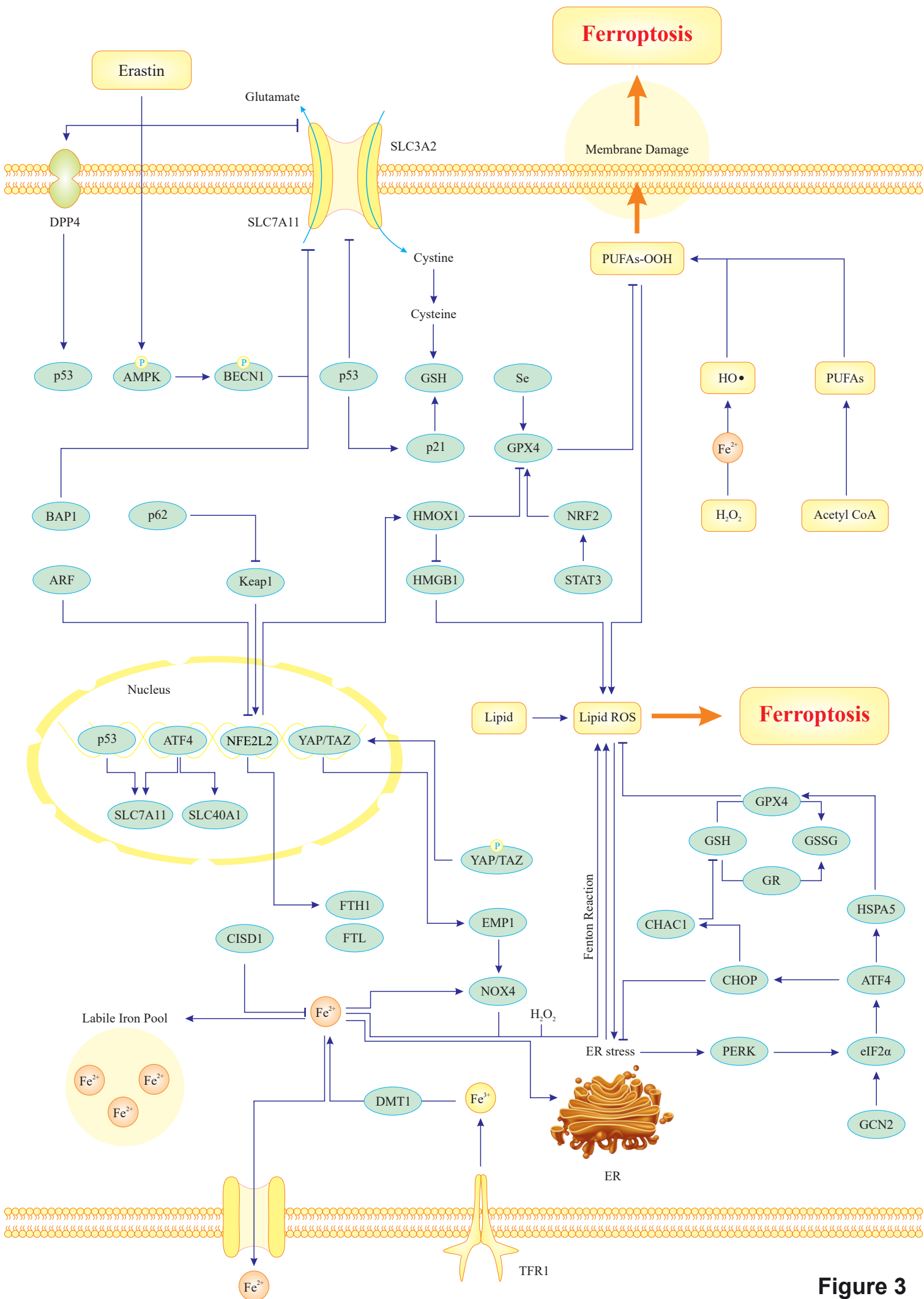


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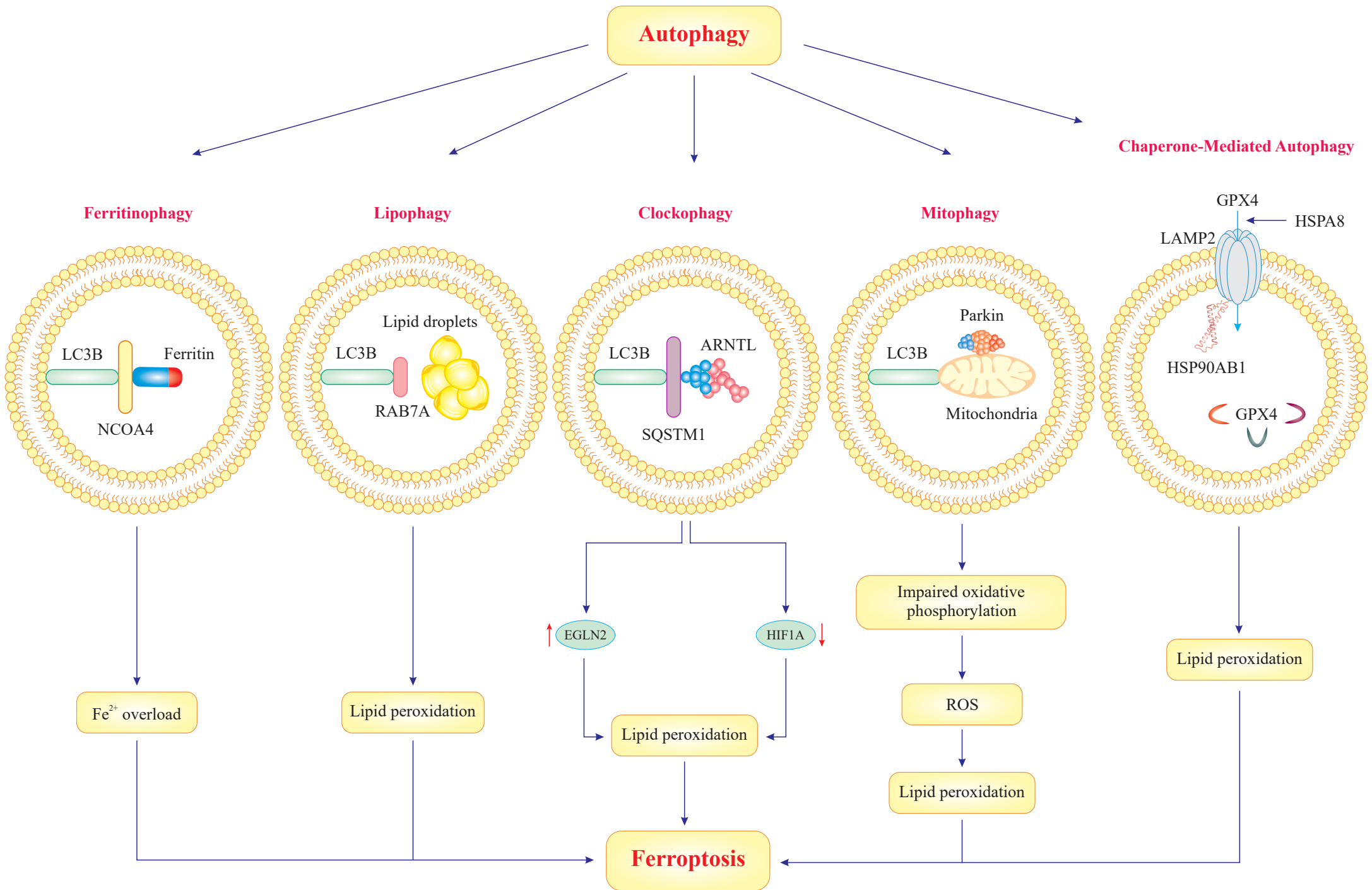


Figure 4

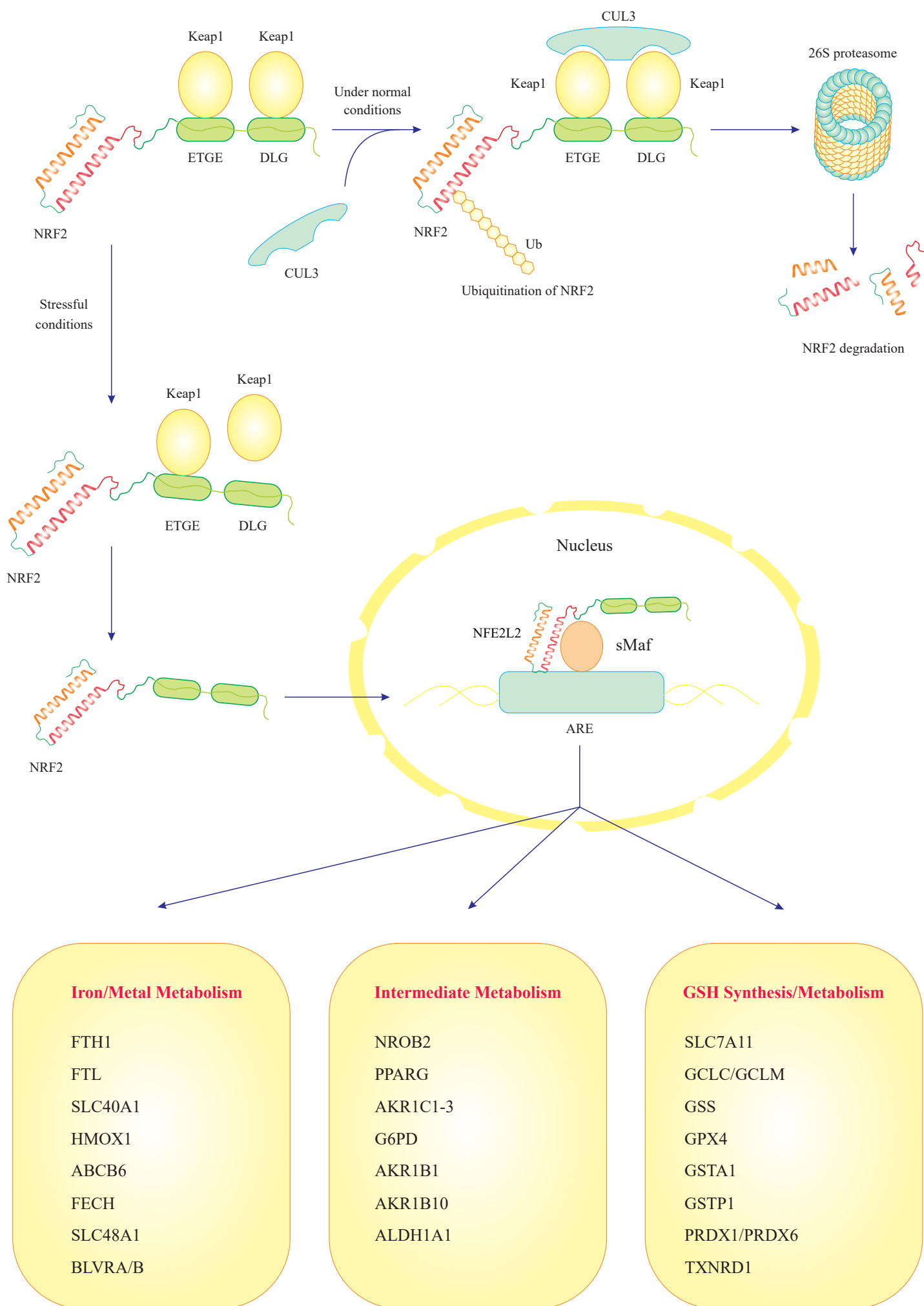


Figure 5

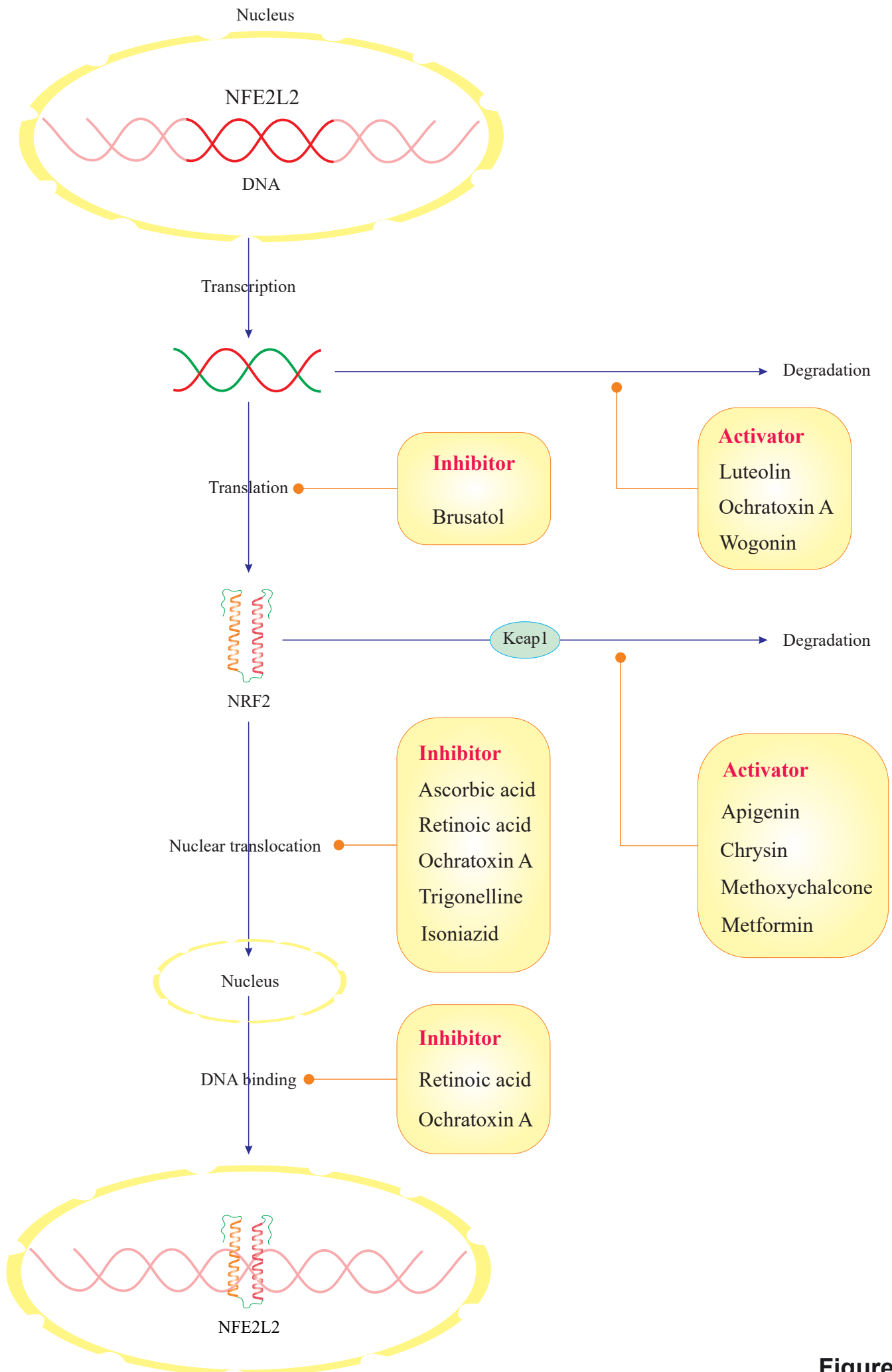


Figure 6