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# REVIEW

## **Ferroptosis in Hepatocellular Carcinoma: Mechanisms and Targeted Therapy**

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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 $\alpha$   
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- $\alpha$  (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- $\alpha$  (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 <sup>1</sup>. Liver cancer ranks the 3<sup>rd</sup> among all cancer  
113 mortalities globally next to lung and colorectal cancers and HCC constitutes 75% of liver cancers  
114 <sup>2</sup>. 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 <sup>3</sup>. **Chronic hepatitis** “(see the Glossary)” resulting from persistent infections with  
117 hepatotropic viruses often ending in HCC <sup>4</sup>. 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 <sup>5,6</sup>. Indeed, **non-alcoholic**  
120 **steatohepatitis (NASH)** is now considered the main risk factor of HCC in developed countries <sup>7</sup>.  
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 <sup>3</sup>. 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 <sup>8</sup>. 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 <sup>9,10</sup>. Cell death can be categorized into either accidental  
127 or regulated <sup>11</sup>. 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 <sup>12</sup>. Reactive oxygen species (ROS) act as the prime drivers of RCD under  
130 impaired cellular antioxidant systems <sup>13</sup>. Broadly speaking, ferroptosis refers to a form of ROS-  
131 mediated RCD, which is executed by dynamic signaling cascades (**Figs 1, 2, 3**) <sup>14</sup>. 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**)<sup>15</sup>. 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<sup>16,17</sup>. 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 ( $\text{Fe}^{2+}$ , absorbable) and ferric iron ( $\text{Fe}^{3+}$ , less soluble)<sup>18,19</sup>. Iron  
145 metabolism is a complex process involving four main phases: absorption, distribution, storage, and  
146 excretion (**Fig 1**)<sup>20</sup>. Both endogenous and exogenous (nutritional) sources supply the iron needed  
147 for maintaining homeostasis<sup>21</sup>. Duodenal mucosal cells absorb nutritional iron to either release it  
148 into circulation or store as ferritin (**Fig 1**)<sup>20</sup>. 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 accumulation<sup>22</sup>. Transferrin is a plasma glycoprotein  
152 that binds and transports circulatory  $\text{Fe}^{3+}$  to various organs<sup>23</sup>. Serum transferrin-bound  $\text{Fe}^{3+}$  is  
153 discerned by TFR1 receptors (encoded by *TFRC*) on cell membranes, mediating endosomal  
154 delivery of  $\text{Fe}^{3+}$  into cytosols (**Figs 1, 2**)<sup>24</sup>. Subsequently,  $\text{Fe}^{3+}$  is reduced to  $\text{Fe}^{2+}$  by endosomal  
155 STEAP3 enzyme, prior to cytoplasmic release via SLC11A2 transporters (**Fig 1**)<sup>25</sup>. Cytosolic  $\text{Fe}^{2+}$



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  $\text{Fe}^{3+}$ )<sup>26</sup>. FTH1 and FTL share  
159 high sequence homology albeit they differ in iron utilization and storage<sup>26</sup>. 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  $\text{Fe}^{2+}$  from its carrier (**Figs 1, 3, 4**)  
162 <sup>27</sup>. Excessive intracellular  $\text{Fe}^{2+}$  can be extruded by the carrier SLC40A1 towards extracellular  
163 space, where it is converted back into  $\text{Fe}^{3+}$ , to balance redox state and iron homeostasis (**Figs 1, 2**)  
164 <sup>28,29</sup>. 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)<sup>30</sup>. Under conditions of iron overload (e.g., **hemochromatosis**) iron  
168 load may exceed the transferrin carrying capacity, thereby, floating in circulation as NTBI<sup>31</sup>. It is  
169 thought that cell surface **ferrireductases** such as CYBRD1 and cell-secreted reductants such as  
170 ascorbate mediate the uptake of NTBI<sup>31</sup>. Mechanistically, ferrireductases and ascorbate convert  
171 NTBI into  $\text{Fe}^{2+}$ , which is subsequently imported into the cytosol via transporters including  
172 SLC39A14, SLC39A8, and SLC11A2 (**Fig 1**)<sup>32</sup>. Ensuing erastin challenge (a ferroptosis inducer),  
173 expression of *SLC11A2* and *TFRC* was upregulated in HT1080 cells<sup>33</sup>, indicating contribution of  
174 NTBI and transferrin-bound iron in ferroptosis induction. Similarly, liver-specific *SLC39A14*  
175 knockout inhibited ferroptosis in mouse livers<sup>34</sup>, 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 ferroptosis<sup>35</sup>. For instance, (i) inhibition and prevention

179 of ferroptotic cell death with iron chelators both *in vivo* and *in vitro* <sup>35</sup>, (ii) ferroptosis induction is  
180 accompanied by elevated intracellular labile iron <sup>36</sup>, and (iii) iron supplementation enhanced  
181 ferroptosis induction upon treatment with erastin <sup>37</sup>. 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 <sup>38,39</sup>. 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**) <sup>40-43</sup>. 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 Fe<sup>2+</sup> and H<sub>2</sub>O<sub>2</sub> (**Figs 1, 2**) <sup>44</sup>. 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 <sup>29,40-42</sup>. 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 <sup>45,46</sup>. 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 <sup>47,48</sup>. Lipid peroxidation of phosphatidyl PUFAs (PUFAs-PL) engages

202 specific enzymes, such as ACSL4 and ALOXs (**Figs 1, 2**)<sup>46,49,50</sup>. ACSL4 catalyzes the synthesis  
203 and enrichment of PUFAs, particularly, arachidonic acid in cell membranes, thereby, acting as a  
204 pro-ferroptotic factor<sup>51</sup>. With regards to the ACSL family, upregulation of ACSL4 is considered  
205 a vital biomarker and driver for ferroptotic death in cancer cells<sup>51</sup>. Moreover, upregulation of  
206 LPCAT3, an essential ACSL4 downstream signal molecule, may foster ferroptosis<sup>52</sup>. ACSL4 and  
207 LPCAT3 participate in incorporation of arachidonic acid into cell membranes, thereby, paving the  
208 way for ferroptosis induction<sup>53,54</sup>. 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 induction<sup>55</sup>.  
211 In the second model, ALOXs enzymes directly catalyze PUFAs oxygenation to drive ferroptosis  
212<sup>56,57</sup>. Also, cytochrome P450 oxidoreductase plays a cardinal role in ferroptosis of cancer cells by  
213 providing ROS for phospholipid peroxidation<sup>58</sup>. Moreover, electron leakage from mitochondrial  
214 electron chain can lead to ROS generation and lipid peroxidation<sup>59</sup>. 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<sup>60,61</sup>. Alternatively, cytochrome P450 oxidoreductase may mediate lipid peroxidation in  
219 ferroptosis in an ALOX-independent manner<sup>62</sup>, whereas lipophagy-mediated degradation of lipid  
220 droplets provides fatty acids for subsequent lipid peroxidation in HCC cells during ferroptosis<sup>62</sup>.  
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**)<sup>63</sup>.  
224 Mechanistically, specific ferroptosis inducers, such as sulfasalazine and erastin ignite ferroptosis

225 via blockade of system  $xc^-$ <sup>37,64</sup>. Of note, sorafenib, an approved drug for HCC treatment, has been  
226 repeatedly evinced to ignite ferroptosis through blockade of system  $xc^-$ <sup>37,64</sup>. 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 ferroptosis<sup>65</sup>.

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 bond<sup>66</sup>. 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*<sup>67,68</sup>. Besides, upregulation of TP53 has been  
236 shown to nullify *SLC7A11* expression, thereby, predisposing to ferroptosis<sup>69</sup>. 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-OOH<sup>70</sup>. 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<sup>71,72</sup>. 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 cells<sup>72</sup>. Also, iron chelators and  
247 ferrostatin-1 (a lipophilic antioxidant) prevented ferroptotic cell death induced by *GPX4* deletion

248 in murine cells <sup>71,73</sup>. 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 <sup>74</sup>. 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<sup>-</sup> to remove toxic phospholipid  
253 hydroperoxide from membranes <sup>72</sup>. Regarding the precise role of lipid peroxidation in ferroptosis,  
254 a decline in the GPX4-GSH antioxidant system provokes ferroptosis <sup>70</sup>. Hence, inhibition of GPX4  
255 by small molecule compounds such as RSL3, FIN56 or ML210, and pharmacological inhibition  
256 of system xc<sup>-</sup> using erastin stimulates ferroptosis (**Figs 1, 2**) <sup>72</sup>. In addition, AIFM2, GCH1 and  
257 DHODH-ignited intracellular antioxidant pathways play an alternative or synergistic role in  
258 combating ferroptotic injury <sup>75</sup>. 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 <sup>76</sup>. 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**) <sup>77</sup>. 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<sup>78-80</sup>. 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<sup>81</sup>. Intriguingly, most proteins involved in the detoxification of lipid peroxidation properties are -  
276 encoded by NRF2 target genes (**Figs 1, 3, 5**)<sup>82</sup>. NRF2-transactivated genes are also heavily  
277 involved in the regulation of iron/heme metabolism<sup>83</sup>. For example, FTL and FTH1, as well as  
278 SLC40A1, are upregulated by NRF2 (**Figs 1, 3, 5**)<sup>84,85</sup>. Moreover, *HMOX1*, *FECH*, *ABCB6*  
279 (critical players in heme biosynthesis), and SLC48A1 (a transporter of heme) are all transactivated  
280 by NRF2 (**Fig 5**)<sup>86-88</sup>. 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<sup>89</sup>. 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**)<sup>89</sup>. 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<sup>89,90</sup>.  
287 Thus, knockdown of *SQSTM1* (encoding p62), *NQO1*, *HMOX1*, *FTH1* or *MT1G* sensitized HCC  
288 cells to ferroptosis upon exposure to sorafenib and erastin<sup>89,90</sup>. 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<sup>89</sup>. 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 <sup>91</sup>. GSTZ1, an enzyme involved in phenylalanine/tyrosine  
296 catabolism, is dysregulated in various malignancies <sup>92</sup>. 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 <sup>93</sup>. Furthermore, inhibition of GPX4 by RSL3 fostered sorafenib-induced ferroptosis in  
300 *Gstz1*<sup>-/-</sup> mice, hinting to the feasibility of a combination therapy with sorafenib and RSL3 against  
301 sorafenib-resistant cells <sup>93</sup>. 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 <sup>94</sup>. 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 <sup>95</sup>. 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 <sup>96</sup>. Activation of the PI3K-AKT1-NRF2 signaling cascade was  
310 suggested to facilitate *ABCC5* upregulation <sup>96</sup>. Moreover, *ABCC5* upregulation promoted  
311 *SLC7A11* expression, downgraded lipid peroxidation and boosted intracellular GSH levels,  
312 resulting in suppressed ferroptosis <sup>96</sup>. Of note, inhibition or downregulation of *ABCC5* promoted  
313 ferroptosis *in vitro* and *in vivo* <sup>96</sup>. 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 <sup>97</sup>. QSOX1 is an enzyme  
318 catalyzing thiol oxidization during protein folding, thus reducing oxygen to hydrogen peroxide <sup>98</sup>.  
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* <sup>97</sup>. Due  
321 to NRF2 inhibition, QSOX1 expression facilitates sorafenib-triggered ferroptosis both *in vitro* and  
322 *in vivo* <sup>97</sup>. 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 <sup>99</sup>.  
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 <sup>100</sup>. Based on the key role of CLTRN in amino acid  
328 transportation through regulation of membrane distribution and solute carrier (SLC) transporters  
329 <sup>101</sup>, these authors postulated a relationship between CLTRN and glutathione metabolism and  
330 ferroptosis <sup>100</sup>. 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* <sup>101</sup>. 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 ferroptosis<sup>102</sup>. Mechanistically, YAP1/TAZ transcriptional factors upregulated  
344 *SLC7A11* in a manner contingent upon TEAD1 activation<sup>102</sup>. *SLC7A11*, a crucial amino acid  
345 transporter (**Fig 1**), favors intracellular glutathione production, and thereby, elicits resistance to  
346 sorafenib-triggered ferroptosis<sup>102</sup>. Moreover, YAP1/TAZ transcriptional factors promoted nuclear  
347 localization and activation of ATF4, which in turn, transactivated *SLC7A11*, conferring resistance  
348 to ferroptosis<sup>102</sup>. 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 $\alpha$  activation, which then resulted in ferroptosis  
355 inhibition through upregulation of *SLC7A11* mRNA<sup>103</sup>. These investigators revealed that  
356 *METTL14* induced N<sup>6</sup>-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*<sup>103,104</sup>. These findings suggest  
359 that hypoxia blocks ferroptosis in HCC cells through activation of the HIF-1 $\alpha$ -*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 $\alpha$  plays an active role in promoting

362 ferroptosis in renal cancers <sup>105,106</sup>. 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) <sup>107</sup>. Such non-coding RNAs participate in tumorigenesis and may also affect  
368 ferroptosis <sup>107</sup>. Circular-interleukin-4 receptor (*circIL4R*) is highly expressed in HCC cells <sup>108</sup>. Xu  
369 and colleagues studied the role of *circIL4R* in HCC progression and ferroptosis and revealed that  
370 *circIL4R* conferred protection against ferroptosis <sup>109</sup>. Knockdown of *circIL4R* elicited ferroptosis  
371 and thereby impeded oncogenesis <sup>109</sup>. 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 <sup>109</sup>. 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 <sup>110</sup>. Further  
379 analysis disclosed that *SLC7A11* and *circ0097009* directly and concurrently bound to microRNA  
380 (*miR*)-1261 <sup>110</sup>. 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 <sup>110</sup>. 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 <sup>111</sup>. *circ0013731* boosted cell growth due to suppressed ferroptosis in HCC cells (QGY-7703,  
387 SMMC-7721) and xenograft models <sup>111</sup>. Mechanistically, *circ0013731* upregulates *SLC7A11* by  
388 direct binding to and sponging *miR-877-3p* <sup>111</sup>. 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 <sup>112</sup>. 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 <sup>112</sup>. 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 <sup>113</sup>. 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) <sup>114</sup>. Thus, abrogation of SPARC expression diminished sorafenib-mediated  
404 killing of these cell lines <sup>114</sup>. Conversely, SPARC overexpression promoted ferroptosis through  
405 excessive oxidative stress, thus, enhancing sorafenib cytotoxicity <sup>114</sup>. 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 <sup>115</sup>. Bioinformatic analyses by Zhang and associates  
409 uncovered that ABCA8 and ABCA9 are markedly downregulated, while ABCA6 is upregulated  
410 in HCC cells <sup>115</sup>. Further analysis showed that ABCB6 participates in the regulation of ferroptosis  
411 <sup>115</sup>. 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 <sup>116</sup>. 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 <sup>117</sup>. Combination of  
418 carbon ion irradiation with sorafenib triggered a mixed-type of apoptotic and ferroptotic cell death  
419 <sup>117</sup>. 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 <sup>118</sup>. S1R is assumed to regulate oxidative stress in an  
425 NRF2-dependent manner <sup>119</sup>, 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 <sup>120</sup>. 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* <sup>120</sup>. Interestingly, NRF2 inhibition promoted S1R expression and conferred protection against

431 ferroptosis <sup>120</sup>. 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 <sup>121</sup>. In  
437 HCC cells, ceruloplasmin blocks ferroptosis by modulating iron homeostasis <sup>122</sup>. Ceruloplasmin  
438 depletion accelerates ferroptosis by increasing intracellular accumulation of Fe<sup>2+</sup> and lipid-  
439 associated ROS in HCC cells under treatment with RSL3 or erastin <sup>122</sup>. Conversely, overexpression  
440 of *CP* prevents ferroptosis <sup>122</sup>. These data indicate that upregulation of *CP*/ceruloplasmin fends off  
441 ferroptosis via reduction of intracellular Fe<sup>2+</sup> <sup>122</sup>. 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 <sup>123</sup>. 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 <sup>124</sup>. 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**) <sup>125</sup>. 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 <sup>126</sup>. Indeed, *G6PD* knockdown resulted in the abatement of tumor weight and  
455 volume <sup>126</sup>. 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 Cisd2 is localized at the ER and mitochondrial outer membrane  
458 (MOM) <sup>127</sup>. Cisd2 serves as a key regulator for cellular processes including intracellular Ca<sup>2+</sup>  
459 homeostasis, mitochondrial function, and ER integrity (**Fig 3**) <sup>127</sup>. It has been evinced that Cisd2  
460 is associated with HCC progression. *Li* and team demonstrated that Cisd2 was overly upregulated  
461 in HCC cells, contributing to disease progression and sorafenib resistance <sup>128</sup>. Thus, *Cisd2*  
462 knockdown reversed sorafenib resistance, reduced viability, and provoked ferroptosis in HCC cells  
463 <sup>128</sup>. Mechanistically, *Cisd2* knockdown triggered excessive Beclin1-dependent autophagy and  
464 ferritinophagy, which spurred ferroptosis in sorafenib-resistant HCC cells <sup>128</sup>. Moreover, genetic  
465 inhibition of Cisd1 favored iron-mediated intramitochondrial lipid peroxidation, contributing to  
466 erastin-induced ferroptosis in human HCC cells <sup>129</sup>. Hence both Cisd1 and Cisd2 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 <sup>130</sup>. Subsequent studies confirmed a role for Nupr1 in suppressing  
471 ferroptosis by reducing mitochondrial oxidative damage in HCC cells <sup>131</sup>. Consequently, the  
472 Nupr1 inhibitor ZZW-115 can increase the sensitivity of pancreatic cancer cells and HCC cells  
473 to ferroptosis <sup>130,131</sup>. 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 <sup>132</sup>. 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 <sup>132</sup>. Notably, CRISPR/Cas9-mediated *miR*-23a-3p knockout boosted the sorafenib  
483 response in HCC cells and orthotopic HCC xenografts <sup>132</sup>. 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 <sup>133</sup>, 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* <sup>134</sup>. PABPC1 was found to interact  
489 with *FAM134B* mRNA, thereby upregulating its translation <sup>134</sup>. *FAM134B* knockdown suppressed  
490 reticulophagy and overtly promoted ferroptosis sensitivity <sup>134</sup>. 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 <sup>135</sup>.  
495 Mechanistically, cytosolic copper accumulation rescued HIF1- $\alpha$  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* <sup>135</sup>. Moreover, a recent  
499 CRISPR/Cas9 screening uncovered a role for PSTK in reducing therapeutic sensitivity of HCC

500 cells <sup>136</sup>. 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* <sup>136</sup>. Similarly, the PSTK inhibitor punicalin reverted sorafenib  
503 resistance of HCC <sup>136</sup>, 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. <sup>137</sup>. 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) <sup>138</sup>. Moreover, combination  
515 treatment of artesunate and sorafenib elevated oxidative stress and ferritinophagy, thereby,  
516 upregulating ferroptosis in Huh7 cells <sup>138</sup>. In this system, artesunate induced activation of  
517 lysosomal **cathepsin B/L**, lipid peroxidation, and ferritinophagy <sup>138</sup>. Pending additional preclinical  
518 confirmation, these findings suggest that artesunate could be utilized in combination with  
519 sorafenib. Also, the anticancer agent atractylodin <sup>139</sup> inhibits invasion, migration, and proliferation  
520 of HCC cells including Hcm and Huh7 <sup>140</sup>. Moreover, atractylodin elevated ROS levels,  
521 downregulated *GPX4* and *FTL*, and upregulated *TFRC* and *ACSL4*, thus contributing to ferroptosis  
522 <sup>140</sup>. Formosanin C is a natural saponin used in Chinese herbal medicine to alleviate inflammation



523 <sup>141</sup>. As described earlier, a rise in iron pool in response to ferritinophagy promotes ferroptosis (**Figs**  
524 **1, 4**) <sup>45</sup>. 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 <sup>142</sup>.  
526 Mechanistically, formosanin C induced NCOA4-dependent ferritinophagy, leading to Fe<sup>2+</sup>  
527 overload and ferroptosis <sup>142</sup>. Moreover, formosanin C triggered ferroptosis and autophagic flux in  
528 HepG2 cells while enhancing *NCOA4* and reducing *FTH1* mRNA levels<sup>143</sup>. Moreover, formosanin  
529 C-induced ferritinophagy triggers ferroptosis in HA59T and HA22T cells <sup>143</sup>, confirming its  
530 potential therapeutic utility. In addition, haloperidol, a drug used for treatment of schizophrenia  
531 <sup>144</sup>, may substantially potentiate sorafenib- or erastin-induced ferroptosis in HCC <sup>145</sup>. At a  
532 relatively low dose, haloperidol effectively triggered intracellular Fe<sup>2+</sup> overload and lipid  
533 peroxidation <sup>145</sup>. 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 <sup>146</sup>. Heteronemin also blocks proliferation of HA59T and HA22T cell lines  
537 through initiation of caspase-dependent apoptosis <sup>16</sup>. Moreover, heteronemin induces ROS  
538 production and GPX4 downregulation, resulting in ferroptotic cell death <sup>16</sup>. Hence, ferroptosis  
539 inhibition attenuated heteronemin-mediated cell death <sup>16</sup>. **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 <sup>89</sup>. 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 <sup>147</sup>. 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 <sup>147</sup>, 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 <sup>148</sup>. 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* <sup>149</sup>. 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) <sup>150</sup>. 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 <sup>151</sup>. 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 HCC<sup>152</sup>. 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)<sup>152</sup>. 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 subtypes<sup>153</sup>. 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 status<sup>153</sup>. 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 HCC<sup>153</sup>. 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 <sup>154</sup>. 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 <sup>154</sup>. 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 <sup>155</sup>. 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 <sup>156</sup>. 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 <sup>157</sup>.

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

### 631 **Competing interests**

632 GK has been holding research contracts with Daiichi Sankyo, Eleor, Kaleido, Lytix  
633 Pharma, PharmaMar, Samsara, Sanofi, Sotio, Tollys, Vascage and Vasculox/Tioma. GK has been

634 consulting for Reithera. GK is on the Board of Directors of the Bristol Myers Squibb Foundation  
635 France. GK is a scientific co-founder of everImmune, Osasuna Therapeutics, Samsara  
636 Therapeutics and Therafast Bio. GK is the inventor of patents covering therapeutic targeting of  
637 aging, cancer, cystic fibrosis and metabolic disorders. The other authors have no conflict of interest  
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639

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#### 643 **Authors' contributions**

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	<sup>147</sup>
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	<sup>16</sup>
Necrostatin-1 (TNF- $\alpha$ inhibitor)	Blocks ferroptosis due to its antioxidant capacity independent of RIPK1 inhibition in HCC cells (SK-HEP-1 and Huh7)	<sup>158</sup>
Solasonine (a glycoalkaloid)	Remarkably suppresses proliferation, invasion, and migration in HepRG and HepG2 cells and in mouse xenograft models via ferroptosis and GPX4 suppression	<sup>159</sup>
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	<sup>160</sup>

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 <sup>161</sup>. 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 <sup>162-164</sup>. In recent years, nanomedicine has participated to the  
671 development of ferroptosis inducers <sup>165,166</sup>. 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 <sup>167</sup>. Alternatively nanomaterials can be developed with  
675 the scope to generate hybrid molecules that combine ferroptosis induction with malignant  
676 cell targeting <sup>167,168</sup>. 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 <sup>169</sup>. 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 <sup>170</sup>. They also conjugated pH-responsive  
682 chitosan to the HMPB surface to boost the biocompatibility of sorafenib <sup>170</sup>. 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* <sup>170</sup>. Mechanistically, nanocomposite ignited excessive ROS generation  
686 and ferroptosis, resulting in HCC shrinkage in mice <sup>170</sup>. 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 <sup>171</sup>.  
689 Moreover, loading MMSNs with sorafenib facilitated specific targeting of the HCC TME



690 <sup>171</sup>. 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 Fe<sup>3+</sup> binds to transferrin, which in turn, binds to TFR1 for  
699 endocytosis. In endosomes, Fe<sup>3+</sup> is converted into Fe<sup>2+</sup> prior to release into the cytosol via  
700 SLC11A2, leading to Fe<sup>2+</sup> 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 Fe<sup>2+</sup> is also exported through  
703 SLC40A1 or stored as Fe<sup>3+</sup> within ferritin. Mitochondrial Fe<sup>2+</sup> overload, a direct result of  
704 cytosolic Fe<sup>2+</sup> overload, triggers ROS generation, thus, promoting lipid peroxidation and  
705 ferroptosis. Moreover, ferritinophagy is another process leading to Fe<sup>2+</sup> 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 adrenolytic acid, which  
718 are key substrates for initiation of lipid peroxidation. Finally, various compounds can target  
719 GPX4, Fe<sup>2+</sup> 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 Fe<sup>2+</sup> 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 **Ferrereductases:** A group of enzymes that mediate the reduction of Fe<sup>3+</sup> to Fe<sup>2+</sup>.

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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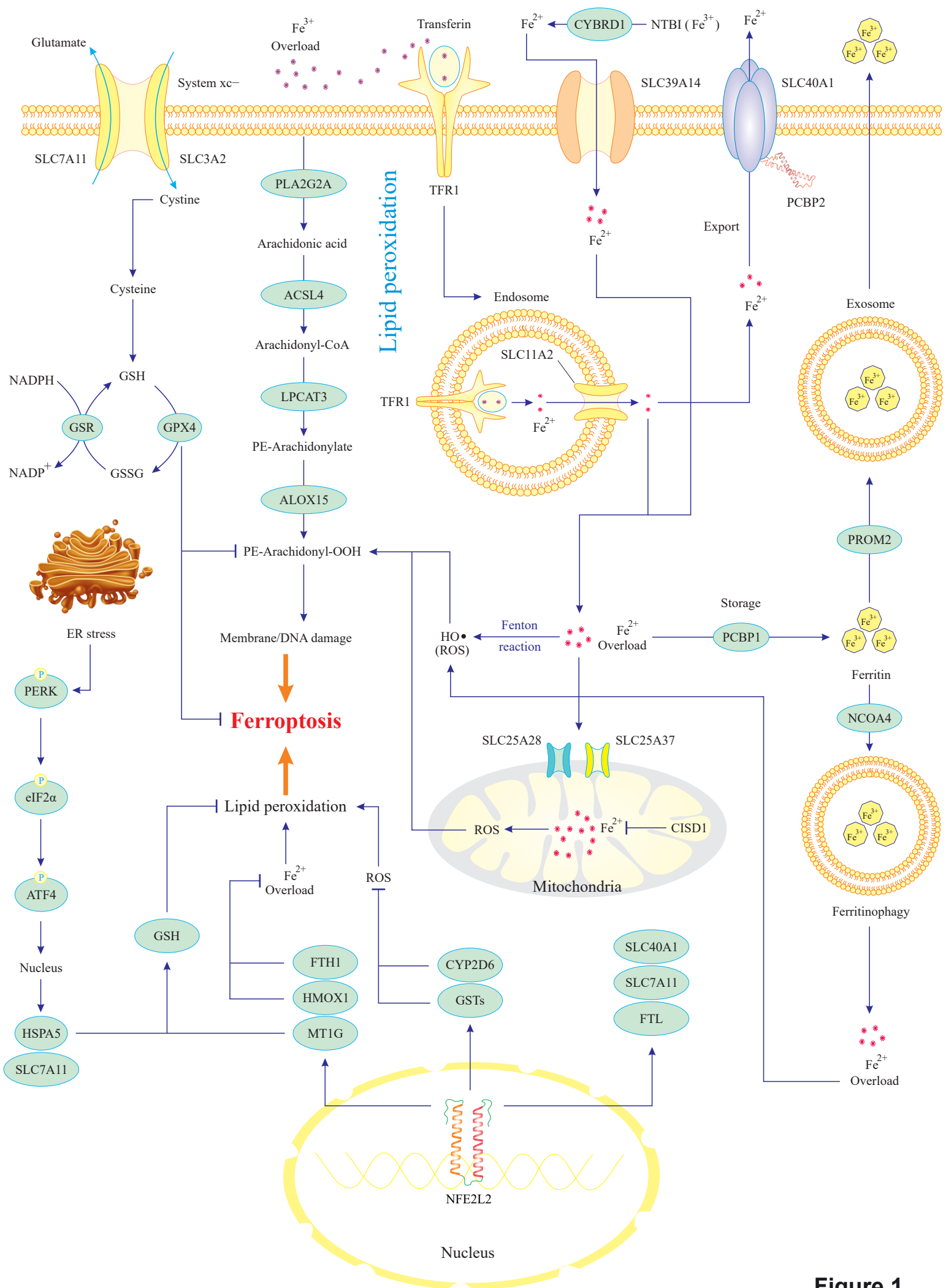
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**Figure 1**

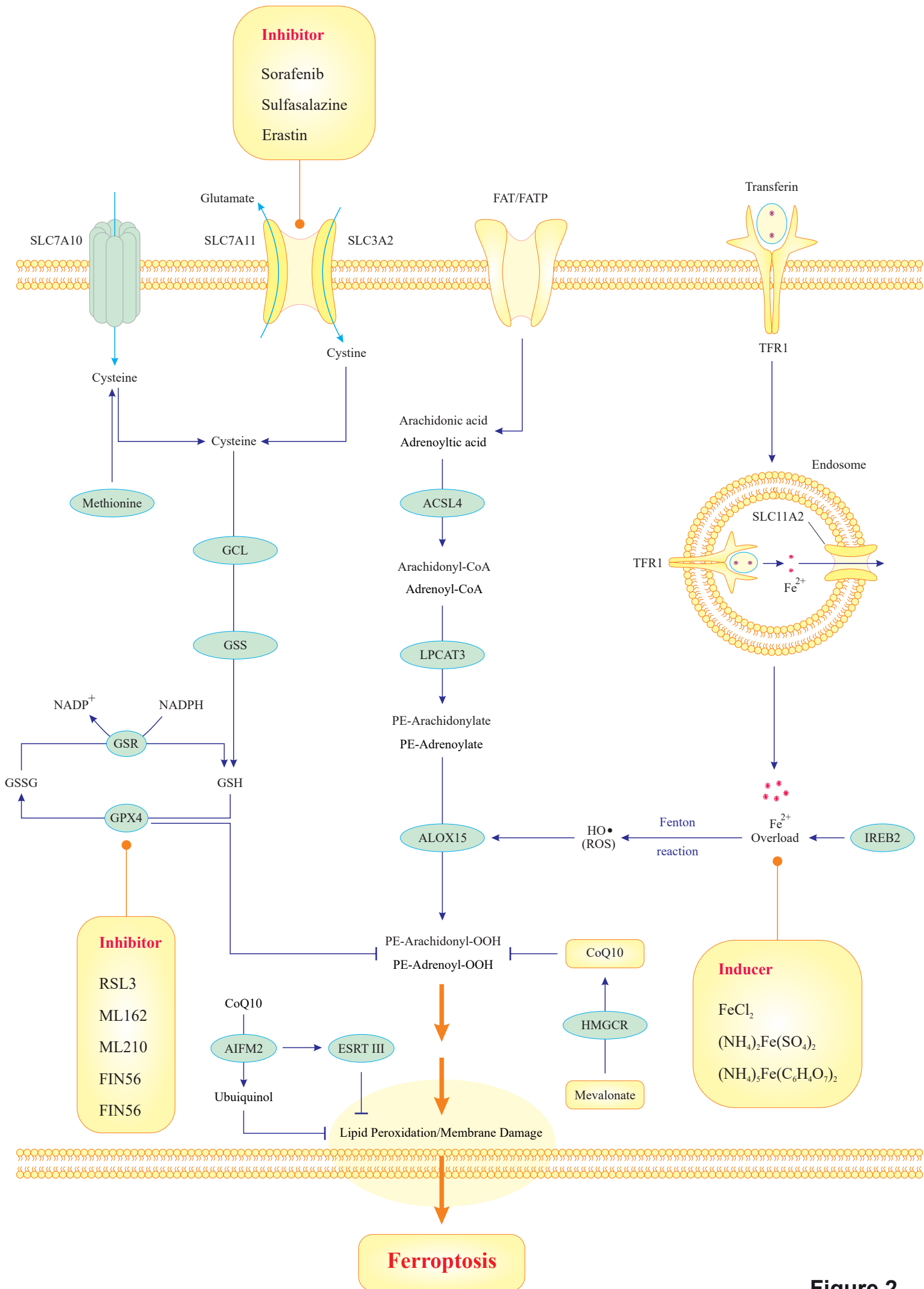
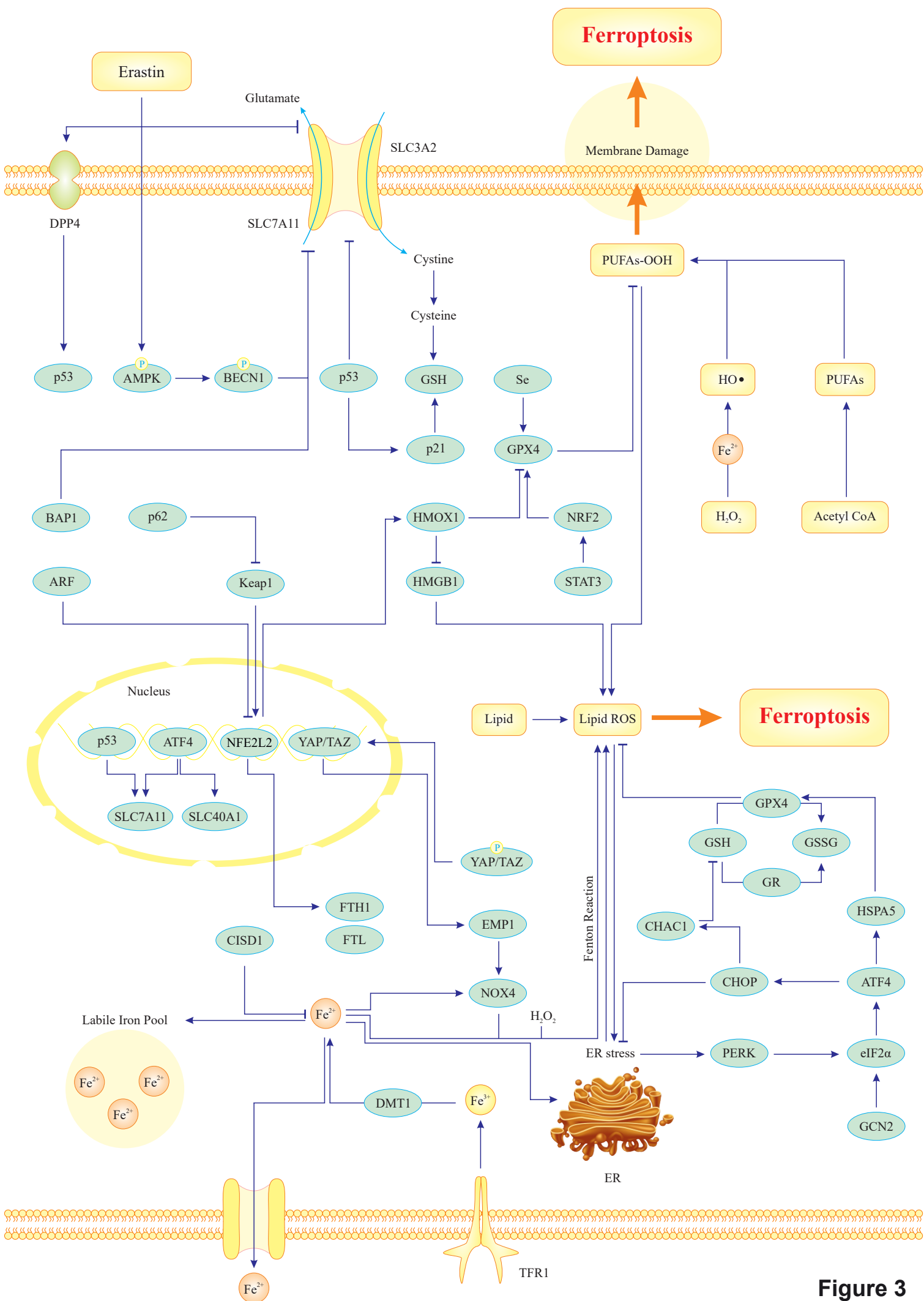
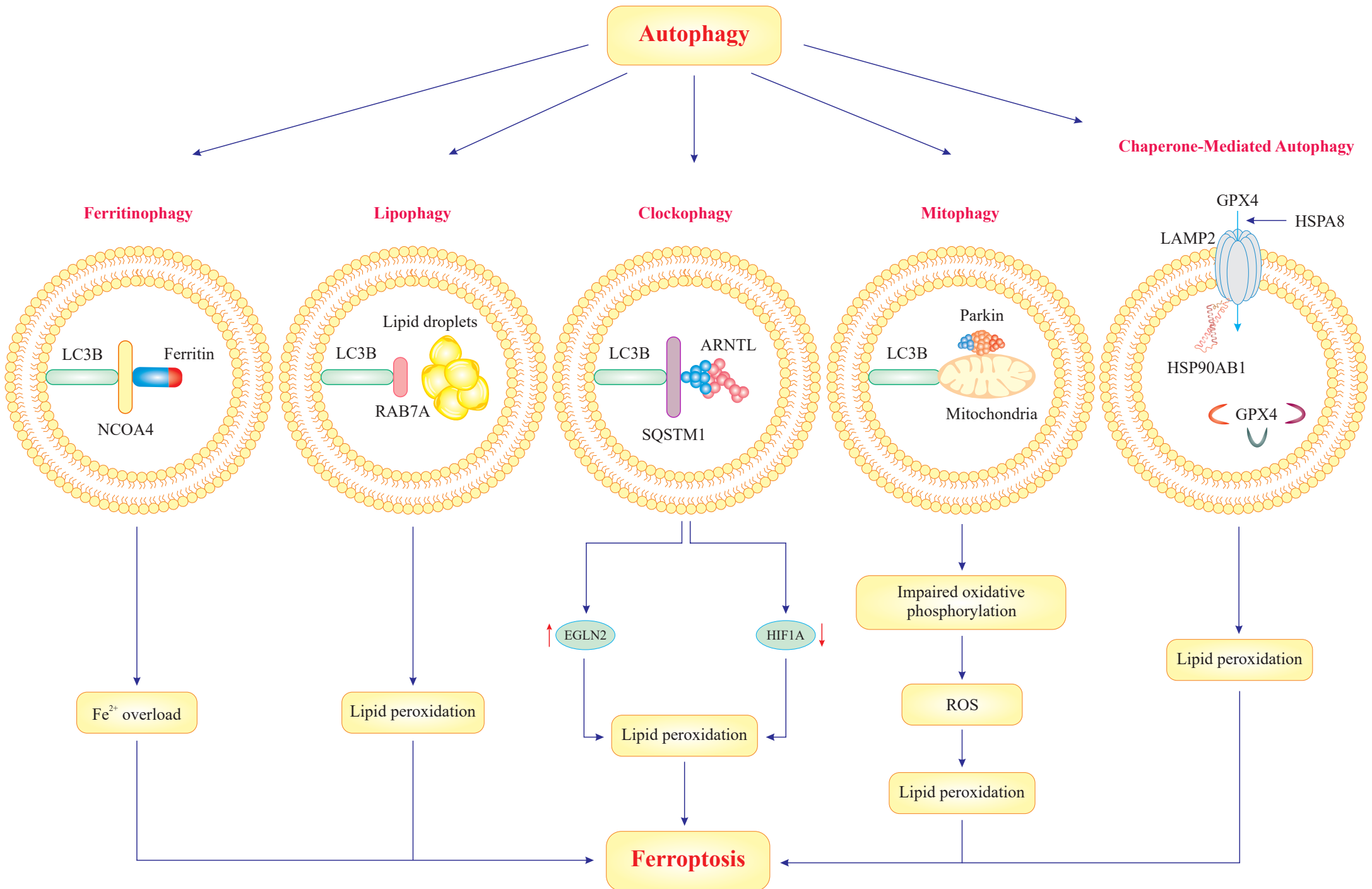


Figure 2

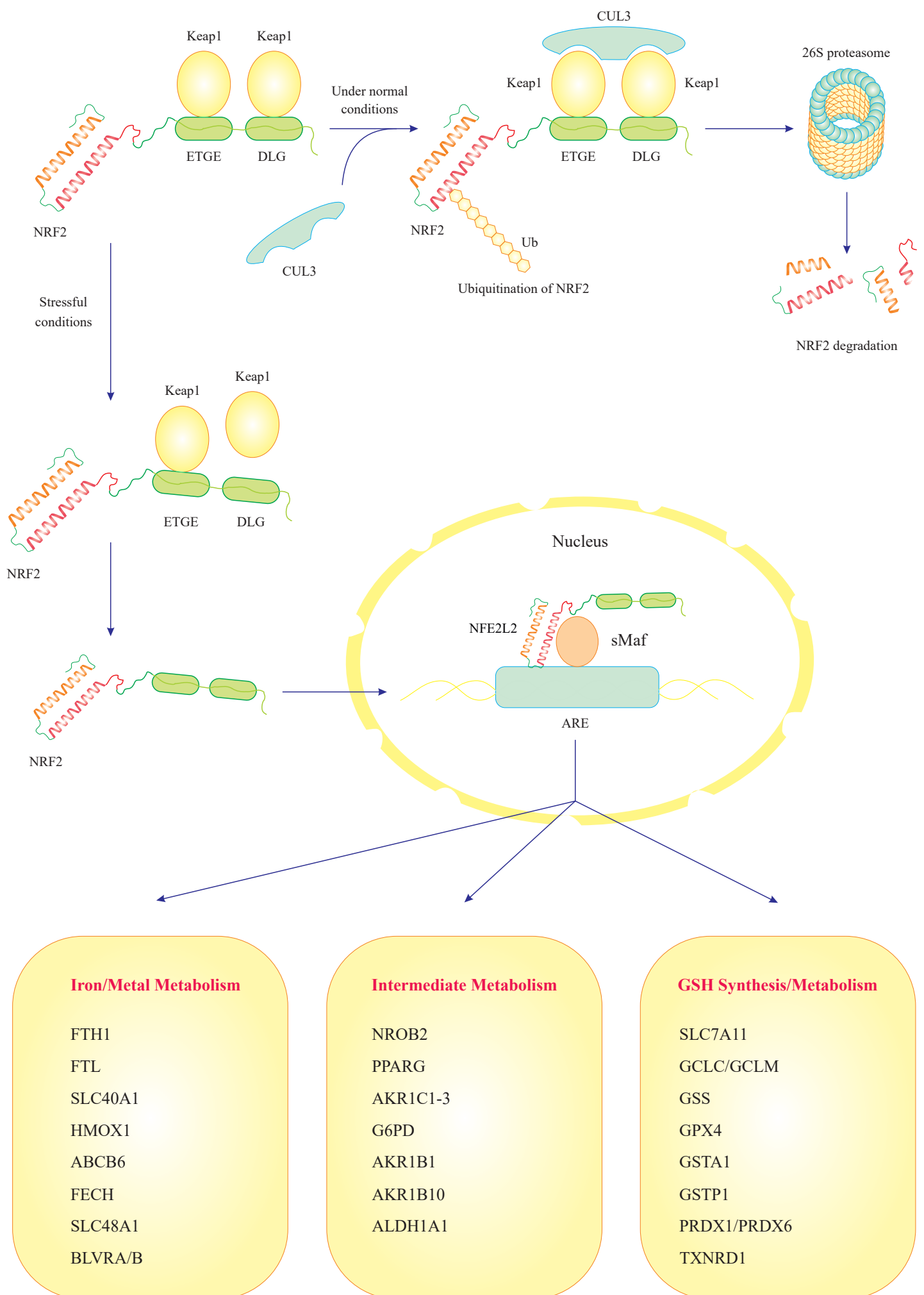


**Figure 3**

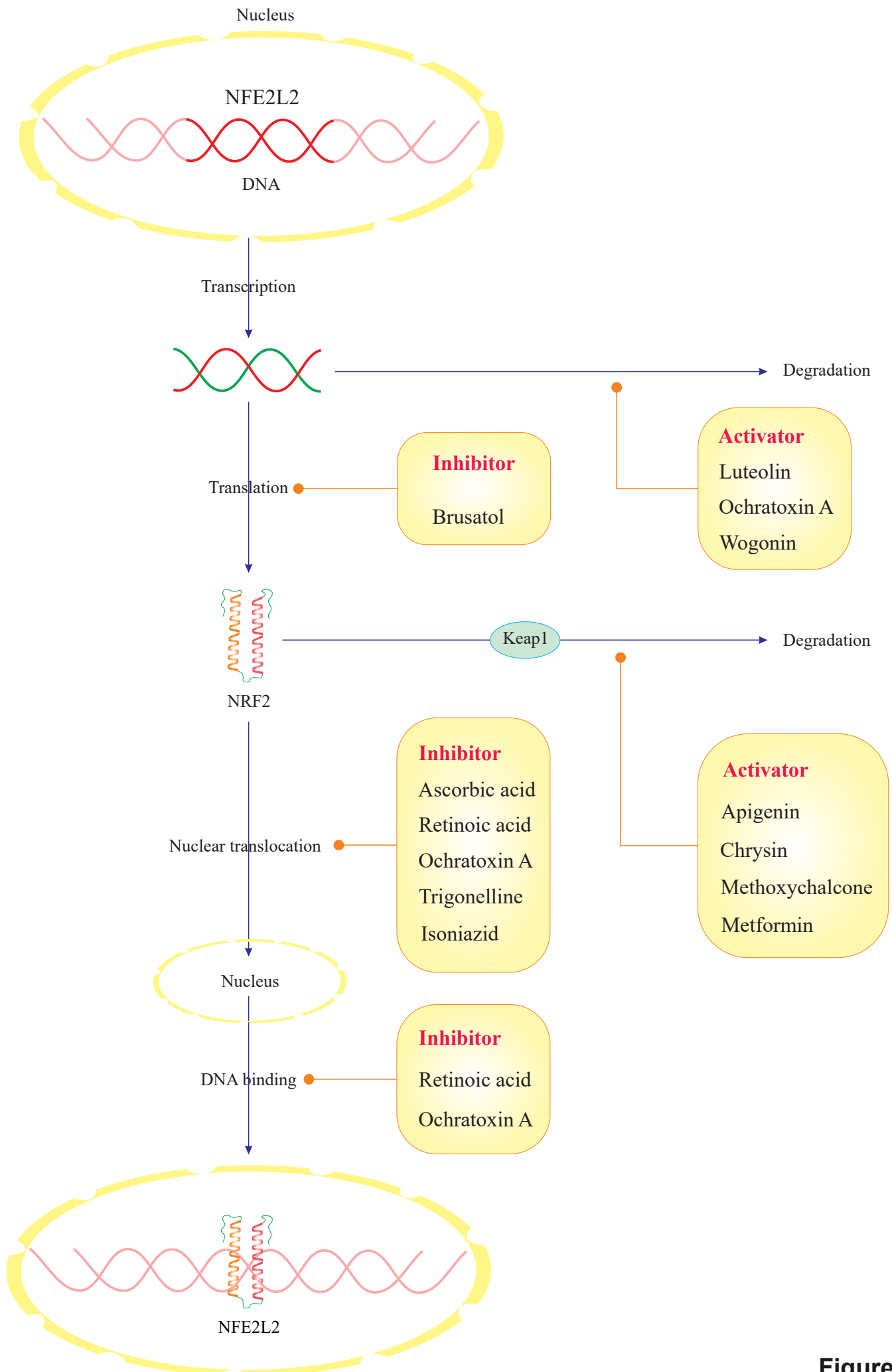




**Figure 4**



**Figure 5**



**Figure 6**