Peer Review File

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Response to Reviewer A

Comment 1: The article addresses a crucial aspect related to cancer, and the potential inclusion of cell death mechanisms, such as ferroptosis, shows promise in the fight against tumors. The article is interesting, and the literature review is well-done; however, I believe that the ferroptosis mechanism, regulated by iron, should be more extensively described in the introduction. Are there studies utilizing the activation of the ferroptosis pathway to kill cancer cells? Are there any literature findings on how this affects normal cells?

Reply 1: First, thank you for your comments and the affirmations of our study. We have added the alterations of ferroptosis in normal and cancer cells, and described the mechanism of ferroptosis in more detail. It has been added to "Specific knockout of the transferrin gene in the hepatocyte of mice with high Fe^{2+} diet increased the possibility of liver fibrosis induced by ferroptosis, and knockout of solute carrier family 39 member 14 (SLC39A14) expression in the ferroptosis pathway or treatment with ferroptosis inhibitors could effectively alleviate liver fibrosis [11]. Insufficient GPX4 promoted ferroptosis including bronchial and kidney epithelial cells and neurons [12]. Taken together, the results suggest that weather tumor cells or normal cells are sensitive to ferroptosis."

Changes in the text: We have modified our text as advised (see Page 2, line 49-54)".

11. Yu Y, Jiang L, Wang H et al. Hepatic transferrin plays a role in systemic iron homeostasis and liver ferroptosis. Blood 2020: 136, 726-739

12. Wenzel S E, Tyurina Y Y, Zhao J et al. Pebp1 wardens ferroptosis by enabling lipoxygenase generation of lipid death signals. Cell 2017: 171, 628-641.e26

Comment 2: In the chapter "Ferroptosis in FH-deficient Tumors," an extensive description of the connections between ferroptosis and FH-deficient tumors has been provided, which adds significant value to the overview. The literature is current and up-to-date; however, I miss a few older references that could have delved into, for example, the mechanisms of cell death.

Reply 2: Thanks for your valuable comments. According to your proposal, we have revised the content of the section "Ferroptosis in FH-deficient Tumors ." Content added the mechanisms of cell death about "Peroxidation of PUFAs on the membrane phospholipids contributes to ferroptosis. Studies have shown that the abundance and localization of PUFAs in cells influence the degree of lipid peroxidation, thus affecting ferroptosis [102, 103, 104] ." and "In addition, cellular experiments have demonstrated that $FH^{-/-}$ tumor cells are more sensitive to ferroptosis inducers and regulate tumor cells growth through multiple pathways. Down regulation of GPX4 activity accelerates tumor cell death through succinylation of GPX4 in $FH^{-/-}$ tumor cells. However, it also protects against ferroptosis by activating NRF2 and GSH activities and reducing Fe^{2+} concentration. Overall, multiple pathways are involved in the inhibition of ferroptosis in $FH^{-/-}$ tumor cells [109] ."

Changes in the text: We have modified our text as advised (see Page 7, line 257-259; Page 8,

line 277-282)".

102. Ursini F.Maiorino M. Lipid peroxidation and ferroptosis: The role of gsh and gpx4. Free Radic Biol Med 2020: 152, 175-185
103. Yuan Y, Zhai Y, Chen J et al. Kaempferol ameliorates oxygen-glucose deprivation/reoxygenation-induced neuronal ferroptosis by activating nrf2/slc7a11/gpx4 axis. Biomolecules 2021: 11,
104. He Y J, Liu X Y, Xing L et al. Fenton reaction-independent ferroptosis therapy via glutathione and iron redox couple sequentially triggered lipid peroxide generator. Biomaterials 2020: 241, 119911
109. Kerins M J, Milligan J, Wohlschlegel J A et al. Fumarate hydratase inactivation in hereditary leiomyomatosis and renal cell cancer is synthetic lethal with ferroptosis induction. Cancer Sci 2018: 109, 2757-2766

Response to Reviewer B

Comment 1: Line 16: ".. ferroptosis is involved in the pathogenesis of FH-deficient tumors ..". Ferroptosis is tumor-suppressive. It does not cause tumor.

Reply 1: Thank you for your comments. We are sorry for our mistake. It has been modified to"Recent studies have indicated that ferroptosis is inhibited the pathogenesis and progression of FH-deficient tumors by regulating lipid and iron metabolism, solute carrier family 7, membrane 11- glutathione - glutathione peroxidase 4 (SCL7A11-GSH-GPX4), nuclear factor-factor 2 (NRF2) / heme oxygenase 1 (HO-1) pathways."

Changes in the text: We have modified our text as advised (see Page 1, line 14-17).

Comment 2: Line 23: "They can induce ferroptosis …". Who are these "They"?

Reply 2: Thank you for your comments. "They" represent "this type of tumor cells".

Changes in the text: We have modified our text as advised (see Page 1, line 24-25).

Comment 3: Line 26: What is "anti-fumarate hydratase-related tumor"?

Reply 3: We apologize for not describing this clearly. It has been modified to "At present, the research on ferroptosis in fumarate hydratase-related tumors is still in the basic experimental stage."

Changes in the text: We have modified our text as advised (see Page 1, line 27-28).

Comment 4: Line 37" Ferroptosis is not caused by high concentration of PUFAs, It is caused by increased oxidation of PUFAs.

Reply 4: Thank you for your comments. It has been modified to " Ferroptosis is mainly caused by increased concentration of intracellular oxidation of polyunsaturated fatty acids (PUFAs), "

Changes in the text: We have modified our text as advised (see Page 1, line 36-37).

Comment 5: Line 70: Define Fenton reaction correctly. It does not generate hydrogen peroxide as claimed.

Reply 5: Thank you for your comments. It has been modified to"Increased Fe^{2+} cause excess

production of ROS, by participating in the H_2O_2 reaction (Fenton reaction), which causing DNA and protein damage, disrupting cell membranes, and causing cell death [27] ."
Changes in the text: We have modified our text as advised (see Page 3, line 75-77).

27. Zhao Z. Iron and oxidizing species in oxidative stress and alzheimer's disease. Aging Med (Milton) 2019: 2, 82-87

Comment 6: Line 72: NRF2 has to translocate from the cytoplasm into nucleus in response to ROS. It is not the other way as stated in the review.
Reply 6: Thank you for your comments. It has been modified to"ROS accumulation promotes the translocation of NRF2 from the cytoplasm to the nucleus, where it activates antioxidant enzymes, such as heme oxygenase 1 (HO-1) , to exert antioxidant effects and inhibit ferroptosis."
Changes in the text: We have modified our text as advised (see Page 3, line 77-80).

Comment 7: Line 79: Does human FH contain iron? Talk about the two classes of iron and then state whether the human FH is class I or class II and then talk about whether or not human FH contains iron.
Reply 7: Thank you for your comments. It has been modified to "FH is widely distributed in living body , one is the dimer containing iron-sulfur clusters (4Fe-4S) , which is oxygen sensitive, heat stable and iron dependent. The second class, tetramers, present in human and other eukaryotic cells, have a molecular weight of approximately 200 kDa and do not carry the cofactor iron."
Changes in the text: We have modified our text as advised (see Page 3, line 94-97).

Comment 8: Lone 83: FH is not involved in urea cycle. It plays a role in the metabolism of fumarate that is released as one of the products of the urea cycle at the step mediated by argininosuccinate lyase.
Reply 8: Thank you for your comments. It has been modified to "In the nucleus, FH is involved in a nonclassical TCA cycle of metabolic-epigenetic circuits [40]."
Changes in the text: We have modified our text as advised (see Page 3, line 100-101).

40. Liu X, Si W, He L et al. The existence of a nonclassical tca cycle in the nucleus that wires the metabolic-epigenetic circuitry. Signal Transduct Target Ther 2021: 6, 375

Comment 9: Line 86: How can one have nonsense mutations as non-hereditary?
Reply 9: Thank you for your comments. It has been modified to "Mutations in FH, which are hereditary, can terminate protein synthesis."
Changes in the text: We have modified our text as advised (see Page 3, line 104).

Comment 10: Line 91: What is "a mutation in the quaternary structure"? Mutations occur only in the primary structure even though mutations can interfere with formation of quaternary structure of multimeric proteins.
Reply 10: Thank you for your comments. It has been modified to "Second, a mutation can interfere with the formation of quaternary structure, can impair FH activity."

Changes in the text: We have modified our text as advised (see Page 3, line 108-109).

Comment 11: Line 96, 97: What is TAC cycle?
Reply 11: Thank you for your comments. This is a typo. It has been modified to "TCA cycle".
Changes in the text: We have modified our text as advised (see Page 4, line 113,114).

Comment 12: Line 97: TCA cycle is not involved just in glucose metabolism. In TCA cycle, acetyl-CoA gets oxidized, but this acetyl-CoA can come from glucose, fatty acids as well as amino acids.
Reply 12: Thank you for your comments. It has been modified to "During the TCA cycle, acetyl-CoA (come from glucose, fatty acids as well as amino acids) is completely oxidized into water and carbon dioxide by cells to release large amounts of energy and produce adenosine-5'-triphosphate (ATP), flavin adenine dinucleotide (FADH2), and produce nicotinamide adenine dinucleotide (NADH) to support different physiological activities."
Changes in the text: We have modified our text as advised (see Page 4, line 114-117).

Comment 13: Line 99: What is NADH2? There is no such thing as NADH2!
Reply 13: Thank you for your comments. This is a typo. It has been modified to "NADH".
Changes in the text: We have modified our text as advised (see Page 4, line 117).

Comment 14: Line 100: Energy is not produced mainly from glucose in all cells. Cardiac myocytes use fatty acids as the major producer of energy.
Reply 14: Thanks for the expert's correction. It has been modified to "Although energy is mainly produced via glucose metabolism in both tumors and normal cells,"
Changes in the text: We have modified our text as advised (see Page 4, line 118).

Comment 15: Line 110: What is NADPH2? There is no such thing as NADPH2!
Reply 15: Thank you for your comments. This is a typo. It has been modified to "NADPH" .
Changes in the text: We have modified our text as advised (see Page 4, line 128).

Comment 16: Line 111: What is epicuticular cell damage?
Reply 16: Thank you for your comments. It has been modified to "This prompts the conversion of oxidated glutathione to its reduced form, protect cells from the damage of ROS."
Changes in the text: We have modified our text as advised (see Page 4, line 129-130).

Comment 17: Line 114: FH is key enzyme in the pentose phosphate pathway? Is this a newly discovered pentose phosphate pathway not known for others?
Reply 17: Thank you for your comments. We don't have a clear description here.It has been modified to "Further, in vitro experiments using UOK262 cell culture have shown that suppression of UOK262 cell proliferation in the absence of phosphogluconate dehydrogenase (PGD) . When the pentose phosphate pathway (PPP) was impaired, and FH is essential for cell growth. "
Changes in the text: We have modified our text as advised (see Page 4, line 131-134).

Comment 18: Line 124: What is the connection between DNA repair and demethylation of DNA? Does fumarate cause hypermethylation or hypomethylation? Where is TET in this section?

Reply 18: Thank you for your comments. It has been modified to "Studies using Western blotting have shown that compared to 2-oxoglutarate, fumarate and succinate can competitively inhibit 2-ketoglutarate dependent dioxygenase (2-OGDD) [44] , which are essential for DNA repair and promotes demethylation of DNA, RNA, and histones. The family of 2-OGDD includes Jumonji C-domain lysine demethylases (JmjC-KDMs), ten-eleven translocation (TET) DNA cytosine-oxidizing enzymes, and others. TET catalyze oxidation of methylated cytosines on DNA, thereby facilitating DNA demethylation [56, 57] . JmjC-KDMs can demethylate on histone tails. DNA hypermethylation contributes to tumorigenesis, which is associated with abnormal gene expression [58] . TET also can inhibition of RNA demethylation enhances tumor cell migration [59, 60] . Taken together, FH deficiency inhibits DNA repair and promotes tumor development. "

Changes in the text: We have modified our text as advised (see Page 4, line 142-151).

44. Ajalla Aleixo M A, Rangel V L, Rustiguel J K et al. Structural, biochemical and biophysical characterization of recombinant human fumarate hydratase. The FEBS journal 2019: 286, 1925-1940

56. Baksh S C.Finley L W S. Metabolic coordination of cell fate by α-ketoglutarate-dependent dioxygenases. Trends in cell biology 2021: 31, 24-36

57. Rasmussen K D.Helin K. Role of tet enzymes in DNA methylation, development, and cancer. Genes & development 2016: 30, 733-50

58. Cheishvili D, Boureau L.Szyf M. DNA demethylation and invasive cancer: Implications for therapeutics. British journal of pharmacology 2015: 172, 2705-15

59. Sulkowski P L, Corso C D, Robinson N D et al. 2-hydroxyglutarate produced by neomorphic idh mutations suppresses homologous recombination and induces parp inhibitor sensitivity. Sci Transl Med 2017: 9,

60. Fitzsimmons C M, Mandler M D, Lunger J C et al. Rewiring of rna methylation by the oncometabolite fumarate in renal cell carcinoma. NAR cancer 2024: 6, zcae004

Comment 19: Line 128: ASL does not generate argininosuccinate. Check the urea cycle before writing this section.

Reply 19: Thank you for your comments. It has been modified to "Citrulline and aspartic acid can be generated to argininosuccinate by argininosuccinate synthetase, and then fumarate and arginine can be catalyzed by argininosuccinate lyase."

Changes in the text: We have modified our text as advised (see Page 5, line 153-154).

This reviewer does not want to go on identifying errors in this manuscript. There are so many errors, grammatical and factual. The authors do not seem to have sufficient background knowledge in the area of the review. In addition, the article has not even been checked for grammar.

Response to Reviewer C

Ferroptosis in an iron-dependent form of regulated cell death that is usually accompanied by large amounts of iron accumulation and lipid peroxidation. Emerging evidence suggests that ferroptosis plays a role in a number of diseases, including cancer; however, the precise underlying molecular mechanisms and its roles in tumorigenesis are unclear. Here, the authors review the role of ferroptosis in cancers with mutations in the Kreb's cycle enzyme fumarate hydratase (FH), with a focus on how FH-deficient tumors inhibit ferroptosis.

Major Comments

Comment 1: The overall organization of the paper makes it challenging to understand the information presented. For example, in lines 38-40, the authors describe a role for ferroptosis in several diseases. However, in lines 40-48, the authors only cite examples from the cancer literature. Only later (lines 49-52) do the authors discuss the importance of cancer. It would be clearer to readers if the introduction were rewritten as follows:

"As ferroptosis is involved in many pathological processes (3, 4), it provides a novel target for preventing and treating related human diseases, including cardiovascular diseases, fibrotic diseases, and cancer. Cancer is the leading cause of human death worldwide (7). However, destroying tumor cells effectively while maintaining normal cells is challenging. As tumor cells contain higher concentrations of Fe2+ ions, it is easier to induce ferroptosis in these cells than in normal cells (8, 9). Fan Yang et al. found that…"

Reply 1: Thank you for your comments. It has been modified to "The mode of ferroptosis-mediated death is unique form that of pyroptosis, autophagy, and programmed cell death. It depends on the intracellular ferrous ions (Fe^{2+}) concentration [1] . Ferroptosis is mainly caused by increased concentration of intracellular oxidation of polyunsaturated fatty acids (PUFAs) , iron metabolism disorders, and imbalance in the oxidative system [2] . As ferroptosis is involved in many pathological processes [3, 4] , it provides a novel target for preventing and treating related human diseases, including cardiovascular diseases, fibrotic diseases, and tumors. Cancer is the leading cause of human death worldwide [5, 6] . However, destroying tumor cells effectively while maintaining normal cells are challenging. As most tumor cells contain higher concentrations of Fe^{2+}, it is easier to induce ferroptosis in these cells than in normal cells [7, 8] . Therefore, ferroptosis can be a potential cancer therapy approach. Fan Yang et al. found that promoting ferroptosis by inhibiting glutathione peroxidase 4 (GPX4) activity and/or promoting glutathione metabolism is effective for treating triple-negative breast cancer. This not only induced tumor cells death but also attenuated tumor drug resistance [9] . Zhuo Gao et al. demonstrated that during lipstatin-1 mediated degradation of nuclear factor-factor 2 (NRF2) , ferroptosis occurs in colorectal cancer cells, which inhibits tumor growth in vivo [10] . Specific knockout of the transferrin gene in the hepatocyte of mice with high Fe^{2+} diet increased the possibility of liver fibrosis induced by ferroptosis, and knockout of solute carrier family 39 member 14 (SLC39A14) expression in the ferroptosis pathway or treatment with ferroptosis inhibitors could effectively alleviate liver fibrosis [11]. Insufficient GPX4 promoted ferroptosis including bronchial and kidney epithelial cells and neurons [12]. Taken together, the results suggest that weather tumor cells or normal cells are sensitive to ferroptosis."

Changes in the text: We have modified our text as advised (see Page 2, line 34-54).

1. Mou Y, Wang J, Wu J et al. Ferroptosis, a new form of cell death: Opportunities and challenges in cancer. J Hematol Oncol 2019: 12, 34
2. Li J, Cao F, Yin H L et al. Ferroptosis: Past, present and future. Cell Death Dis 2020: 11, 88
3. Chen X, Kang R, Kroemer G et al. Broadening horizons: The role of ferroptosis in cancer. Nature reviews. Clinical oncology 2021: 18, 280-296
4. Jiang X, Stockwell B R.Conrad M. Ferroptosis: Mechanisms, biology and role in disease. Nat Rev Mol Cell Biol 2021: 22, 266-282
5. Siegel R L, Miller K D, Wagle N S et al. Cancer statistics, 2023. CA: a cancer journal for clinicians 2023: 73, 17-48
6. Wang X, Shen Y, Wan X et al. Oncolytic virotherapy evolved into the fourth generation as tumor immunotherapy. Journal of translational medicine 2023: 21, 500
7. Ke K, Li L, Lu C et al. The crosstalk effect between ferrous and other ions metabolism in ferroptosis for therapy of cancer. Frontiers in oncology 2022: 12, 916082
8. Wang D, Tang L, Zhang Y et al. Regulatory pathways and drugs associated with ferroptosis in tumors. Cell Death Dis 2022: 13, 544
9. Yang F, Xiao Y, Ding J H et al. Ferroptosis heterogeneity in triple-negative breast cancer reveals an innovative immunotherapy combination strategy. Cell Metab 2023: 35, 84-100.e8
10. Gao Z, Jiang J, Hou L et al. Lysionotin induces ferroptosis to suppress development of colorectal cancer via promoting nrf2 degradation. Oxid Med Cell Longev 2022: 2022, 1366957
11. Yu Y, Jiang L, Wang H et al. Hepatic transferrin plays a role in systemic iron homeostasis and liver ferroptosis. Blood 2020: 136, 726-739
12. Wenzel S E, Tyurina Y Y, Zhao J et al. Pebp1 wardens ferroptosis by enabling lipoxygenase generation of lipid death signals. Cell 2017: 171, 628-641.e26.

Comment 2: On lines 17-18 authors claim that FH-deficient cells have lower levels of Fe2+. However, on lines 50-51 the authors claim that tumor cells contain higher levels of Fe2+ making ferroptosis induction easier in tumor cells then healthy cells. These two pieces of information are logically inconsistent. If FH-deficient cells have lower levels of free iron making them resistant to ferroptosis and therefore the use of ferroptosis induction a poor strategy in this cancer subtype altogether. This discrepancy should be addressed.

Reply 2: Thank you for your comments. It has been modified to "While the Fe^{2+} content is significantly lower in FH-deficient tumor cells, than that in normal cells. It is promising to promote ferroptosis by increasing the concentration of Fe^{2+} in cells to achieve the purpose of tumor treatment". and "As most tumor cells contain higher concentrations of Fe^{2+}, it is easier to induce ferroptosis in these cells than in normal cells [7, 8] . Therefore, ferroptosis can be a potential cancer therapy approach."

Changes in the text: We have modified our text as advised (see Page 1, line 17-19; Page 2, line 41-43).

7. Ke K, Li L, Lu C et al. The crosstalk effect between ferrous and other ions metabolism in ferroptosis for therapy of cancer. Frontiers in oncology 2022: 12, 916082

8. Wang D, Tang L, Zhang Y et al. Regulatory pathways and drugs associated with ferroptosis in tumors. Cell Death Dis 2022: 13, 544

Comment 3: On lines 61-62 the authors state that triggering ferroptosis in FH-deficient cells may prove a promising approach to cancer treatment. The authors then proceed to outline many ways in which FH deficient cells are resistant to ferroptosis induction. This seems logically inconsistent. This discrepancy should be addressed.

Reply 3: Thank you for your comments. By understanding the specific mechanism of ferroptosis in FH-related tumors, it is possible to treat FH-related tumors. It has been modified to "As the survival, metastasis, and drug-resistance mechanisms of tumor cells are closely related to ferroptosis [35, 36, 37] , regulating ferroptotic signaling pathways can control tumors growth."

Changes in the text: We have modified our text as advised (see Page 3, line 89-91).

35. Hänggi K.Ruffell B. Cell death, therapeutics, and the immune response in cancer. Trends in cancer 2023: 9, 381-396
36. Wang Y, Wu X, Ren Z et al. Overcoming cancer chemotherapy resistance by the induction of ferroptosis. Drug resistance updates : reviews and commentaries in antimicrobial and anticancer chemotherapy 2023: 66, 100916
37. Wang Y, Wang Y, Pan J et al. Ferroptosis, necroptosis, and pyroptosis in cancer: Crucial cell death types in radiotherapy and post-radiotherapy immune activation. Radiotherapy and oncology : journal of the European Society for Therapeutic Radiology and Oncology 2023: 184, 109689

Comment 4: On Line 64, this description of what ferroptosis is feels confusing and lacking critical pieces of information. Ferroptosis is a Fe2+ dependent, distinct form of cell death, induced by the inhibition of key regulators cell including but not limited to SLC7A11, GPX4, and FSP1 that leads to uncontrolled lipid peroxidation of polyunsaturated fatty acids.

Reply 4: Thank you for your comments. It has been modified to "Ferroptosis is a recently discovered non-apoptotic cell death program, which is catalyzing the lipid peroxidation of unsaturated fatty acids in the cell membrane leading to cell death , and is closely related to Fe^{2+} content."

Changes in the text: We have modified our text as advised (see Page 3, line 70-72).

Comment 5: On lines 73-75 it is not clear what the authors mean when they state that inducing ferroptosis inhibits tumor development by inducing synthesis of NRF2 which is canonically thought of as a mechanism of tumor chemotherapeutic resistance.

Reply 5: Thank you for your comments. It has been modified to "According to the existing literature, promoting ferroptosis can inhibit tumors development through different pathways, such as consume intracellular GSH, and induce ferroptosis in non-small cell lung cancer cells [32] ; It promotes lipid peroxidation and fosters ferroptosis by inducing ROS accumulation, when gemcitabine is used to treat pancreatic cancer [33] ; increasing Fe^{2+} in the labile iron pool (LIP) induces ferroptosis in breast cancer cells [34]."

Changes in the text: We have modified our text as advised (see Page 3, line 84-89).

32. Guo J, Xu B, Han Q et al. Ferroptosis: A novel anti-tumor action for cisplatin. Cancer research and treatment 2018: 50, 445-460
33. Jiang Y X, Chen J Z, Li M W et al. The combination of bacillus thuringiensis and its engineered strain expressing dsrna increases the toxicity against plutella xylostella. Int J Mol Sci 2021: 23,
34. Zhang J, Yang J, Zuo T et al. Heparanase-driven sequential released nanoparticles for ferroptosis and tumor microenvironment modulations synergism in breast cancer therapy. Biomaterials 2021: 266, 120429

Comment 6: On lines 86-88 the authors state that "few FH mutations have been reported to be non-pathogenic." Indeed, multiple mutations are known to exist that are classified as benign or likely benign. The authors can find a complete up-to-date list of all known mutations on the FH mutation database (https://doi.org/10.1186/1471-2350-9-20). Furthermore, work by Heather Christofk's lab has demonstrated that FH tetramerization is necessary for enzymatic activity; however, multimerization status alone cannot predict pathogenicity (https://doi.org/10.1101/2022.08.15.504023).

Reply 6: Thank you for your comments. It has been modified to "Mutations in FH, which are hereditary, can terminate protein synthesis[41] . Mutations in FH genes are not necessarily pathogenic [42] , but FH tetramerization is necessary for enzymatic activity [43] ."

Changes in the text: We have modified our text as advised (see Page 3, line 104-106).

41. Mai S, Yanagi T, Shimano M et al. Case of hereditary leiomyomatosis and renal cell cancer showing multiple cutaneous leiomyomas harboring a recurrent nonsense mutation in the fumarate hydratase gene. J Dermatol 2022: 49, e42-e43
42. Bayley J P, Launonen V.Tomlinson I P. The fh mutation database: An online database of fumarate hydratase mutations involved in the mcul (hlrcc) tumor syndrome and congenital fumarase deficiency. BMC medical genetics 2008: 9, 20
43. Wilde B R, Chakraborty N, Matulionis N et al. Fh variant pathogenicity promotes purine salvage pathway dependence in kidney cancer. Cancer discovery 2023: 13, 2072-2089

Comment 7: On lines 121-124, the authors discuss the role of succinate and fumarate in cells, and then cite a paper (reference 44) that discusses the role of IDH mutations. Mutations to IDH1/2 lead to de novo production of 2-hydroxyglutarate. The authors should include 2-hydroxygulararate in their list of metabolites that can inhibit 2-oxoglutarate-dependent dioxygenases, and ensure correct citation of references to studies discussing the role of succinate and fumarate accumulation.

Reply 7: Thank you for your comments. It has been modified to "Studies using Western blotting have shown that compared to 2-oxoglutarate, fumarate and succinate can competitively inhibit 2-ketoglutarate dependent dioxygenase (2-OGDD) [44] , which are essential for DNA repair and promotes demethylation of DNA, RNA, and histones. The family of 2-OGDD includes Jumonji C-domain lysine demethylases (JmjC-KDMs), ten-eleven translocation (TET) DNA cytosine-oxidizing enzymes, and others. TET catalyze oxidation of methylated cytosines on DNA, thereby facilitating DNA demethylation [56, 57] . JmjC-KDMs can demethylate on histone tails.

DNA hypermethylation contributes to tumorigenesis, which is associated with abnormal gene expression [58] . TET also can inhibition of RNA demethylation enhances tumor cell migration [59, 60] . Taken together, FH deficiency inhibits DNA repair and promotes tumor development."
Changes in the text: We have modified our text as advised (see Page 4, line 142-151).

44. Ajalla Aleixo M A, Rangel V L, Rustiguel J K et al. Structural, biochemical and biophysical characterization of recombinant human fumarate hydratase. The FEBS journal 2019: 286, 1925-1940
56. Baksh S C.Finley L W S. Metabolic coordination of cell fate by α-ketoglutarate-dependent dioxygenases. Trends in cell biology 2021: 31, 24-36
57. Rasmussen K D.Helin K. Role of tet enzymes in DNA methylation, development, and cancer. Genes & development 2016: 30, 733-50
58. Cheishvili D, Boureau L.Szyf M. DNA demethylation and invasive cancer: Implications for therapeutics. British journal of pharmacology 2015: 172, 2705-15
59. Sulkowski P L, Corso C D, Robinson N D et al. 2-hydroxyglutarate produced by neomorphic idh mutations suppresses homologous recombination and induces parp inhibitor sensitivity. Sci Transl Med 2017: 9,
60. Fitzsimmons C M, Mandler M D, Lunger J C et al. Rewiring of rna methylation by the oncometabolite fumarate in renal cell carcinoma. NAR cancer 2024: 6, zcae004

Comment 8: On lines 121-124, the authors discuss the role of succinate and fumarate accumulation in cells, suggesting that this decreases cellular viability. However, in numerous cancers, including HLRCC, accumulation of fumarate leads to more invasive cells and a transition to a more mesenchymal phenotype. This discrepancy should be addressed.
Reply 8: Thank you for your comments. It has been modified to " Increased concentrations of succinate and fumarate, particularly the latter, are genetically toxic to cells, dramatically leading to the activation of oncogenic [55] ."
Changes in the text: We have modified our text as advised (see Page 4, line 140-142).

55. Wentzel J F, Lewies A, Bronkhorst A J et al. Exposure to high levels of fumarate and succinate leads to apoptotic cytotoxicity and altered global DNA methylation profiles in vitro. Biochimie 2017: 135, 28-34

Comment 9: On lines 124-125, the authors say that 2-oxoglutarate dependent enzymes are critical for DNA repair in HLRCC. This is a limited view. Enzymes in the 2OGDD family are also critical for histone demethylation (see https://www.nature.com/articles/nature19353) , RNA demethylation (https://www.biorxiv.org/content/10.1101/2023.04.10.536262v1) , and prolyl-hydroxylase.
Reply 9: Thank you for your comments. After carefully reading the above literatures, I re-outlined introduction. It has been modified to "Studies using Western blotting have shown that compared to 2-oxoglutarate, fumarate and succinate can competitively inhibit 2-ketoglutarate dependent dioxygenase (2-OGDD) [44] , which are essential for DNA repair and promotes demethylation of DNA, RNA, and histones. The family of 2-OGDD includes Jumonji C-domain lysine demethylases (JmjC-KDMs), ten-eleven translocation (TET) DNA cytosine-

oxidizing enzymes, and others. TET catalyze oxidation of methylated cytosines on DNA, thereby facilitating DNA demethylation [56, 57] . JmjC-KDMs can demethylate on histone tails. DNA hypermethylation contributes to tumorigenesis, which is associated with abnormal gene expression [58] . TET also can inhibition of RNA demethylation enhances tumor cell migration [59, 60] . Taken together, FH deficiency inhibits DNA repair and promotes tumor development."
Changes in the text: We have modified our text as advised (see Page 4, line 142-151).

44. Ajalla Aleixo M A, Rangel V L, Rustiguel J K et al. Structural, biochemical and biophysical characterization of recombinant human fumarate hydratase. The FEBS journal 2019: 286, 1925-1940
56. Baksh S C.Finley L W S. Metabolic coordination of cell fate by α-ketoglutarate-dependent dioxygenases. Trends in cell biology 2021: 31, 24-36
57. Rasmussen K D.Helin K. Role of tet enzymes in DNA methylation, development, and cancer. Genes & development 2016: 30, 733-50
58. Cheishvili D, Boureau L.Szyf M. DNA demethylation and invasive cancer: Implications for therapeutics. British journal of pharmacology 2015: 172, 2705-15
59. Sulkowski P L, Corso C D, Robinson N D et al. 2-hydroxyglutarate produced by neomorphic idh mutations suppresses homologous recombination and induces parp inhibitor sensitivity. Sci Transl Med 2017: 9,
60. Fitzsimmons C M, Mandler M D, Lunger J C et al. Rewiring of rna methylation by the oncometabolite fumarate in renal cell carcinoma. NAR cancer 2024: 6, zcae004

Comment 10: On line 176-180, the authors cite three mutations found on cBioPortal. Evidence from the FH mutation database (https://doi.org/10.1186/1471-2350-9-20) would suggest that other mutations occur more frequently.
Reply 10: Thank you for your comments. We mainly investigated the relationship between FH mutation sites and related tumors. We read the literature you recommended and found that R190H is a common mutation in RCC. It has been modified to "R190H mutation is the most commonly described FH variant in Renal cell cancer (RCC) [42]"
Changes in the text: We have modified our text as advised (see Page 6, line 201-202).

42. Bayley J P, Launonen V.Tomlinson I P. The fh mutation database: An online database of fumarate hydratase mutations involved in the mcul (hlrcc) tumor syndrome and congenital fumarase deficiency. BMC Med Genet 2008: 9, 20

Comment 11: On lines 223-227, Ferroptosis does not disrupt the lipid membrane due to increased PUFA's. Rather, the existing PUFA's in the membrane are oxidized leading to lipid peroxide formation and propagation. The levels of PUFA's in the membrane can determine the relative sensitivity to undergoing ferroptosis but PUFA increase prior to ferroptosis induction is not a part of this mechanism canonically.
Reply 11: Thank you for your comment. It has been modified to "Lipids are essential for maintaining cell function and membrane structure [101] . During ferroptosis, the lipid bilayer is disrupted due to increased peroxidation PUFAs, affecting normal cell membrane functions. GPX4 reduces cytotoxic peroxides to their corresponding alcohols, and regulates lipid

peroxidation. Inhibition of GPX4 activity can exacerbate lipid peroxidation and promote ferroptosis [102] . contrarily, it suppresses ferroptosis.

In FH-deficient cells, the acetyl-CoA left over after the TCA cycle is used for fatty acid synthesis. Peroxidation of PUFAs on the membrane phospholipids contributes to ferroptosis. Studies have shown that the abundance and localization of PUFAs in cells influence the degree of lipid peroxidation, thus affecting ferroptosis [102, 103, 104] . Simultaneously, lipid peroxidation can directly cooperate with GPX4 synthesis, which eventually inhibits ferroptosis (Figure 1C) [105] . In conclusion, to varying degrees, metabolic pathways can influence the effect of ferroptosis on tumor cells."

Changes in the text: We have modified our text as advised (see Page 7, line 251-262).

101. Rochette L, Dogon G, Rigal E et al. Lipid peroxidation and iron metabolism: Two corner stones in the homeostasis control of ferroptosis. Int J Mol Sci 2022: 24,

102. Ursini F.Maiorino M. Lipid peroxidation and ferroptosis: The role of gsh and gpx4. Free radical biology & medicine 2020: 152, 175-185

103. Yuan Y, Zhai Y, Chen J et al. Kaempferol ameliorates oxygen-glucose deprivation/reoxygenation-induced neuronal ferroptosis by activating nrf2/slc7a11/gpx4 axis. Biomolecules 2021: 11,

104. He Y J, Liu X Y, Xing L et al. Fenton reaction-independent ferroptosis therapy via glutathione and iron redox couple sequentially triggered lipid peroxide generator. Biomaterials 2020: 241, 119911

105. Li D, Wang Y, Dong C et al. Cst1 inhibits ferroptosis and promotes gastric cancer metastasis by regulating gpx4 protein stability via otub1. Oncogene 2023: 42, 83-98

Comment 12: In lines 233-234, the authors say that lipid peroxidation-induced ferropotosis has not yet been studied in HLRCC. This is incorrect. Please see this paper, which explores ferroptosis in HLRCC. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6125459/

Reply 12: Thank you for your comment. It has been modified to "In addition, cellular experiments have demonstrated that $FH^{-/-}$ tumor cells are more sensitive to ferroptosis inducers and regulate tumor cells growth through multiple pathways. Down regulation of GPX4 activity accelerates tumor cell death through succinylation of GPX4 in $FH^{-/-}$ tumor cells. However, it also protects against ferroptosis by activating NRF2 and GSH activities and reducing Fe^{2+} concentration. Overall, multiple pathways are involved in the inhibition of ferroptosis in $FH^{-/-}$ tumor cells [109]. "

Changes in the text: We have modified our text as advised (see Page 8, line 277-282).

109. Kerins M J, Milligan J, Wohlschlegel J A et al. Fumarate hydratase inactivation in hereditary leiomyomatosis and renal cell cancer is synthetic lethal with ferroptosis induction. Cancer Sci 2018: 109, 2757-2766

Minor Comments

Comment 13: Please double check and correct italics throughout (e.g. et al. in vitro, de novo, etc)

Reply 13: Thank you for your comment. It has been modified.
Changes in the text: We have modified our text as advised (see Page 2, line 44; Page 2, line 47; Page 4, line 132; Page 5, line 159 and so on.).

Comment 14: Please double check correct superscript and subscript notation throughout (e.g. H2O2, Fe2+, etc)
Reply 14: Thank you for your comment. It has been modified to "Fe^{2+}".
Changes in the text: We have modified our text as advised (see Page 1, line 17; Page 1, line 19; Page 2, line 35; Page 2, line 42; and so on.).

Comment 15: In multiple locations, tricarboxylic acid cycle (TCA) is abbreviated incorrectly as TAC.
Reply 15: Thank you for your comment. It has been modified to "TCA".
Changes in the text: We have modified our text as advised (see Page 3, line 97; Page 3, line 100; Page 4, line 101 and so on).

Comment 16: Please define terms and abbreviations when they are first used (e.g. nuclear factor erythroid 2 related factor 2 (NRF2); Heme oxygenase 1 (HO-1), etc)
Reply 16: Thank you for your comment. It has been modified to "nuclear factor erythroid 2 related factor 2 (NRF2)"; "Heme oxygenase 1 (HO-1)".
Changes in the text: We have modified our text as advised (see Page 1, line 17; Page 1, line 17).

Comment 17: There is a typo on line 106 (duke should be corrected to due)
Reply 17: Thank you for your comment. It has been modified to "due".
Changes in the text: We have modified our text as advised (see Page 4, line 124).

Comment 18: There is a typo on line 121 (letter should be corrected to latter)
Reply 18: Thank you for your comment. It has been modified to "latter".
Changes in the text: We have modified our text as advised (see Page 4, line 141).

Comment 19: There is a typo on line 224 (isdisprupted should be 2 words)
Reply 19: Thank you for your comment. It has been modified to " is disrupted".
Changes in the text: We have modified our text as advised (see Page 7, line 252).