nature portfolio

Peer Review File

Targeting FSP1 triggers ferroptosis in lung cancer

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This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Referee #1

(Remarks to the Author)
This study aimed to investigate whether ferroptosis serves as a barrier to tumorigenesis and how it can be exploited for therapeutic purposes. To address this, the authors conducted a loss-of-function analysis of two key ferroptosis regulators, GPX4 and FSP1, in both a mouse lung cancer model and a transplantation model. Their findings revealed that both GPX4 and FSP1 promote tumorigenesis and cancer progression. Moreover, inhibiting FSP1 demonstrated significant therapeutic benefit in the mouse model. Consistently, high FSP1 expression was correlated with poor patient prognosis. The study concludes that targeting FSP1 to induce ferroptosis represents a promising therapeutic strategy for lung cancer.

The discovery of FSP1's essential contribution to tumorigenesis is highly novel. The experimental design and methodologies are both highly original and robust. The conclusions drawn are well-supported by the presented data, though the mechanistic aspects could be further strengthened.

Several questions and comments need to be addressed.

Major points:

1) In the autochthonous KP LUAD tumor model, CRISPR knockout of Fsp1 or Gpx4 resulted in a reduced tumor burden (Figure 2f and 2g). However, the following sentences suggest that the total number of tumors remained unchanged (Extended Data Figure 2c). Does this imply that the tumors generated after Fsp1 or Gpx4 knockout are smaller in size? If so, it would be valuable to evaluate and compare the size of individual tumors.

2) Figures 2h and Extended Data Figure 2c illustrate that both Gpx4 and Fsp1 are essential for malignant progression, which is quite intriguing. The authors further confirmed that cell proliferation and apoptotic cell death are unaffected by the loss of Gpx4 or Fsp1 (Extended Data Figures 2d and 2e). How does the inhibition of lipid peroxidation mediated by GPX4 and FSP1 contribute to the malignant progression of cancer? It remains to be clarified whether this effect is due to the avoidance of cell death or if other molecular mechanisms related to the regulation of lipid peroxidation are at play.

3) FSP1 is critical for tumorigenesis independent of GPX4; however, in an in vitro setting, FSP1 seems to be dispensable. Additional experiments suggest that FSP1 exerts its pro-tumorigenic effects in a cell-autonomous manner. What could be the molecular mechanism underlying these intriguing findings? Why is GPX4 unable to compensate for the loss of FSP1 in vivo? Data presented in Figure 2d appear to indicate a growth advantage for tumors with high FSP1 expression. What are the functional differences between GPX4 and FSP1 in vivo concerning tumor progression? Does the overexpression of GPX4 in an FSP1-deficient condition reverse tumorigenesis? Conversely, what effect does overexpressing FSP1 in a GPX4-deficient condition have?

Minor points
Extended Data 3i and 3j are not referred in the text.
Extended Data 4a is not referred in the text.

Referee #2

(Remarks to the Author)
This is a manuscript by Wu et al where they have demonstrated targeting FSP1 as a therapeutic strategy in KRAS-driven lung cancer. By CRISPR-Cas9 gene editing, they noted that 1) Fsp1 deletion phenocopies Gpx4 KO in KP LUAD GEMM, 2) Fsp1 loss is sufficient to induce ferroptosis and reduce tumor burden even in the presence of Gpx4, 3) ferroptosis inhibition-genetically, pharmacologically, and dietarily- restores KP lung tumor growth, and 4) icFSP1 effectively inhibits tumor growth in vivo. The authors found FSP1 is an independent target for lung tumors and FSP1inhibition as a novel therapeutic strategy to improve prognosis of the KRAS-driven lung cancer patients. in lung cancer patients. However, given the aims and scope of the journal (suitable for wider public), the manuscript would fit more appropriately into cancer-specific journals (e.g., Nature Cancer).
1) More mechanistic study: authors claim that Fsp1 loss reduces KRAS-driven lung tumor growth, and FSP1 levels are associated with patient outcome. It would be important to investigate 1) how oncogenic KRAS mutation can induce FSP1 expression, 2) if not, how KRAS mutation can create the environment where FSP1 is important for KRAS cancer growth. Also, it has been well known that concurrent mutations of KRAS and KEAP1 or KRAS and LKB1 increase oxidative stress more so than KP oncogenotype. I am wondering if the role of FSP1 (or GPX4) is more critical for molecular subtypes of KRAS-driven lung tumors.

2) It would be worth checking if it is KRAS-dependent or more general phenomenon by comparing to other lung cancer models with different driver mutations.

3) While KP LUAD GEMM is a wonderful tool to study tumorigenesis and tumor aggressiveness, it cannot give us information regarding the variants of KRAS mutations. In other words, it is unclear whether it is a KRAS G12D specific phenotype or other KRAS mutant-lung tumors would show the similar results. KRAS mutant PDXes might be good for the validation study.

4) It has been reported that pathologically activated neutrophils, known as polymorphonuclear (PMN) myeloid-derived suppressor cells (MDSCs), undergo spontaneous ferroptosis, which contributes to immune suppression in cancer (PMID: 36385526). Given the important roles of ferroptosis both in tumor and tumor microenvironment, it would be important to investigate whether systemic FSP inhibition impacts tumor growth and how FSP1 inhibition in the TME affects FSP1 inhibition-mediated tumor reduction.

Referee #3

(Remarks to the Author)
Induction of ferroptosis through the inhibition of cellular defense systems (i.e., GPX4 and FSP1) has been proposed as a strategy to kill therapy-resistant cancer cells. However, whether this is a viable therapeutic approach remains an open question.

In the current manuscript, the authors explore the potential to target GPX4 and FSP1 in lung cancer. Genetic depletion of GPX4 or FSP1 are sufficient to suppress lung tumor growth. The effect of GPX4 depletion was not surprising given the numerous studies showing its essentiality in many types of cancer, though the findings in this immunocompetent GEM model are a nice addition to these reports. The surprising finding was that FSP1 depletion strongly suppressed tumor growth. This finding is in contrast to in vitro data, where knockout of FSP1 or inhibition of FSP1 does not trigger ferroptosis on their own. Similar findings are observed with both the GEM model and a complementary orthotopic xenograft model. The authors further show that FSP1 expression increases with the stage of lung cancer and FSP1 expression correlates with poor patient prognosis. Loss of FSP1 results in increased H&E staining, indicative of lipid peroxidation. The H&E staining and tumor suppression are reversed by treatment with known ferroptosis inhibitors such as Lip1 and vitamin E. Finally, the authors find that the FSP1 targeting molecule icFSP1 increases the survival of mice in an orthotopic lung tumor model.

This is a very nice and important study that is the first to demonstrate the benefit of single agent targeting of FSP1 in cancer, uncovering a ferroptosis vulnerability that is present in vivo but not in vitro. The study is carefully performed and complementary data from multiple models support the conclusions. The data presented address a key question in the field and the findings with these sophisticated mouse models are exciting and have important therapeutic implications for targeting FSP1 and ferroptosis in cancer. There are some comments that should be addressed.

1) Overexpression of GPX4 had a mild effect on the resistance to RSL3-induced ferroptosis (extended fig 1f). In contrast, sodium selenite (Na2SeO3) addition completely blocked RSL3-induced ferroptosis (extended fig 1g). The difference is perhaps unexpected and seems to suggest that Na2SeO3 is protecting cells from ferroptosis through a mechanism independent of GPX4. Please explain further.

2) icFSP1 treatment extended the survival of the mice bearing orthotopic lung tumors. Did this treatment affect tumor size? Is this reversed by Lip1?

3) icFSP1 is a unique “inhibitor” in that it acts by triggering the phase separation of FSP1. In contrast to other FSP1 inhibitors and genetic deletion of FSP1, icFSP1 has been found to have some single agent activity in vitro (PMID: 37380771). This

calls into question the selectivity of the molecule or whether it might be acting through a gain of toxicity rather than loss of FSP1 activity. Would you see a similar effect using a more traditional inhibitor of FSP1 activity?

Minor comments

1) A statement is made regarding the importance of GPX4 in regulating tumor growth - “However, its importance in regulating tumor growth remains unknown.” (lines 92-92) This seems to be a bit of an overstatement given the number of studies exploring GPX4 in cancer. Some amount of refining of this statement would be helpful.

Version 1:

Reviewer comments:

Referee #1

(Remarks to the Author)
In the revised version of the manuscript, questions and concerns from this reviewer have been appropriately addressed. This reviewer has no more comments.

Referee #2

(Remarks to the Author)
In this revision, the authors have added significant and important data that strengthen the paper. All of my concerns have been addressed.

Referee #3

(Remarks to the Author)
This revised manuscript presents a compelling and timely contribution to our understanding of ferroptosis as a tumor-suppressive mechanism in vivo. The authors have addressed all of my prior concerns in a thoughtful and thorough manner. Importantly, the revised version strengthens the manuscript with new experimental data that further substantiates the key claims and adds mechanistic depth, particularly regarding the in vivo effects of FSP1 inhibition.

The central finding, that lung adenocarcinoma (LUAD) tumors are highly dependent on FSP1 for suppressing ferroptosis, is very exciting. While GPX4 has been well-documented as a key ferroptosis suppressor in cancer, FSP1 has generally been viewed as a secondary or redundant player based on in vitro studies. This work convincingly overturns that assumption by demonstrating that genetic ablation of Fsp1, even in the absence of GPX4 loss, profoundly limits tumor progression in immunocompetent genetically engineered mouse models (GEMMs) and orthotopic xenografts.

The new data included in the revision are particularly compelling. The authors now show that treatment with the FSP1 inhibitor icFSP1 significantly reduces tumor burden in vivo, an effect that is reversed by co-treatment with the ferroptosis inhibitor Liproxstatin-1. This directly links the therapeutic benefit of icFSP1 to ferroptotic cell death. The inclusion of additional treatment arms and bioluminescence imaging enhances the rigor and clarity of the in vivo findings. Moreover, the use of a mutant FSP1 allele (Q319K), which resists icFSP1-induced phase separation, provides an elegant genetic control that strengthens the conclusion that icFSP1 acts through on-target inhibition of FSP1.

In sum, this is an outstanding study that addresses a fundamental question in cancer biology, whether inhibiting FSP1 has potential therapeutic value in cancer as a single agent target. The work is technically rigorous, an important advance for the field, and therapeutically relevant.

I enthusiastically support publication.

Nature manuscript 2024-08-17738
Response to Reviewers

We would like to thank all the reviewers for their insightful comments and constructive suggestions to improve the studies detailed in our manuscript. We appreciate the overall positive reviews and critical points raised by each reviewer. Accordingly, we have addressed the reviewers' major questions and minor points. Our point-by-point responses are written below in red (reviewer comments in black).

Referee #1 (Remarks to the Author):

This study aimed to investigate whether ferroptosis serves as a barrier to tumorigenesis and how it can be exploited for therapeutic purposes. To address this, the authors conducted a loss-of-function analysis of two key ferroptosis regulators, GPX4 and FSP1, in both a mouse lung cancer model and a transplantation model. Their findings revealed that both GPX4 and FSP1 promote tumorigenesis and cancer progression. Moreover, inhibiting FSP1 demonstrated significant therapeutic benefit in the mouse model. Consistently, high FSP1 expression was correlated with poor patient prognosis. The study concludes that targeting FSP1 to induce ferroptosis represents a promising therapeutic strategy for lung cancer.

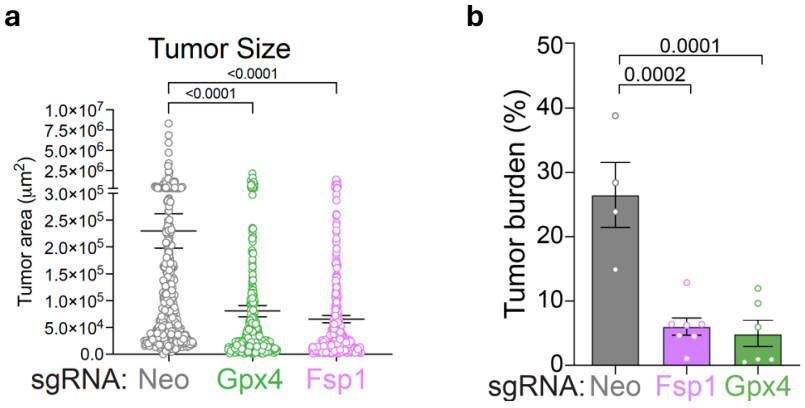
The discovery of FSP1's essential contribution to tumorigenesis is highly novel. The experimental design and methodologies are both highly original and robust. The conclusions drawn are well-supported by the presented data, though the mechanistic aspects could be further strengthened.

Several questions and comments need to be addressed.

We thank the reviewer for highlighting the novelty of this work. We agree that elucidation of the underlying mechanisms for FSP1's requirement in lung tumorigenesis would further strengthen the study, and we have performed several additional experiments regarding this, detailed below.

Major points:
1) In the autochthonous KP LUAD tumor model, CRISPR knockout of Fsp1 or Gpx4 resulted in a reduced tumor burden (Figure 2f and 2g). However, the following sentences suggest that the total number of tumors remained unchanged (Extended Data Figure 2c). Does this imply that the tumors generated after Fsp1 or Gpx4 knockout are smaller in size? If so, it would be valuable to evaluate and compare the size of individual tumors.

We thank the reviewer for pointing this out. Tumor burden was calculated as the total tumor area relative to the total lung tissue area, so indeed tumors generated after Fsp1 or Gpx4 knockout are smaller in size relative to control KP LUAD tumors. This is evident when the data is reanalyzed with the area of individual tumors as a single data point (panel a below, Ext. Data Fig. 3c). However, it is important to keep in mind that this quantification of tumor size reflects only a single cross-sectional "snapshot" of the tumor-bearing lung tissue from each GEMM, which is inevitably dependent on where the tumor grows within the lung parenchyma and which section of tissue is captured for H&E staining. This is internally controlled for when calculating tumor burden given the normalization to the total lung tissue. Thus, we typically prefer to show total tumor burden per mouse (panel b below, Fig. 2f), as each GEMM is infected with the same number of tumor-initiating lentiviral particles, which may more appropriately reflect true biological differences in tumorigenesis across different knockout groups.



(a) *Ext. Data Fig. 3c:* Tumor area of KP LUAD tumors with knockout of either control (Neo), Fsp1, or Gpx4, where each data point represents a single tumor. **(b)** *Fig. 2f*: Tumor burden quantification of KP LUAD tumors with knockout of either control (Neo), Fsp1, or Gpx4, where each data point represents a single mouse infected with the same number of tumor-initiating lentiviral particles. Tumor burden is calculated as total tumor area normalized to total lung tissue.

2) Figures 2h and Extended Data Figure 2c illustrate that both Gpx4 and Fsp1 are essential for malignant progression, which is quite intriguing. The authors further confirmed that cell proliferation and apoptotic cell death are unaffected by the loss of Gpx4 or Fsp1 (Extended Data Figures 2d and 2e). How does the inhibition of lipid peroxidation mediated by GPX4 and FSP1 contribute to the malignant progression of cancer? It remains to be clarified whether this effect is due to the avoidance of cell death or if other molecular mechanisms related to the regulation of lipid peroxidation are at play.

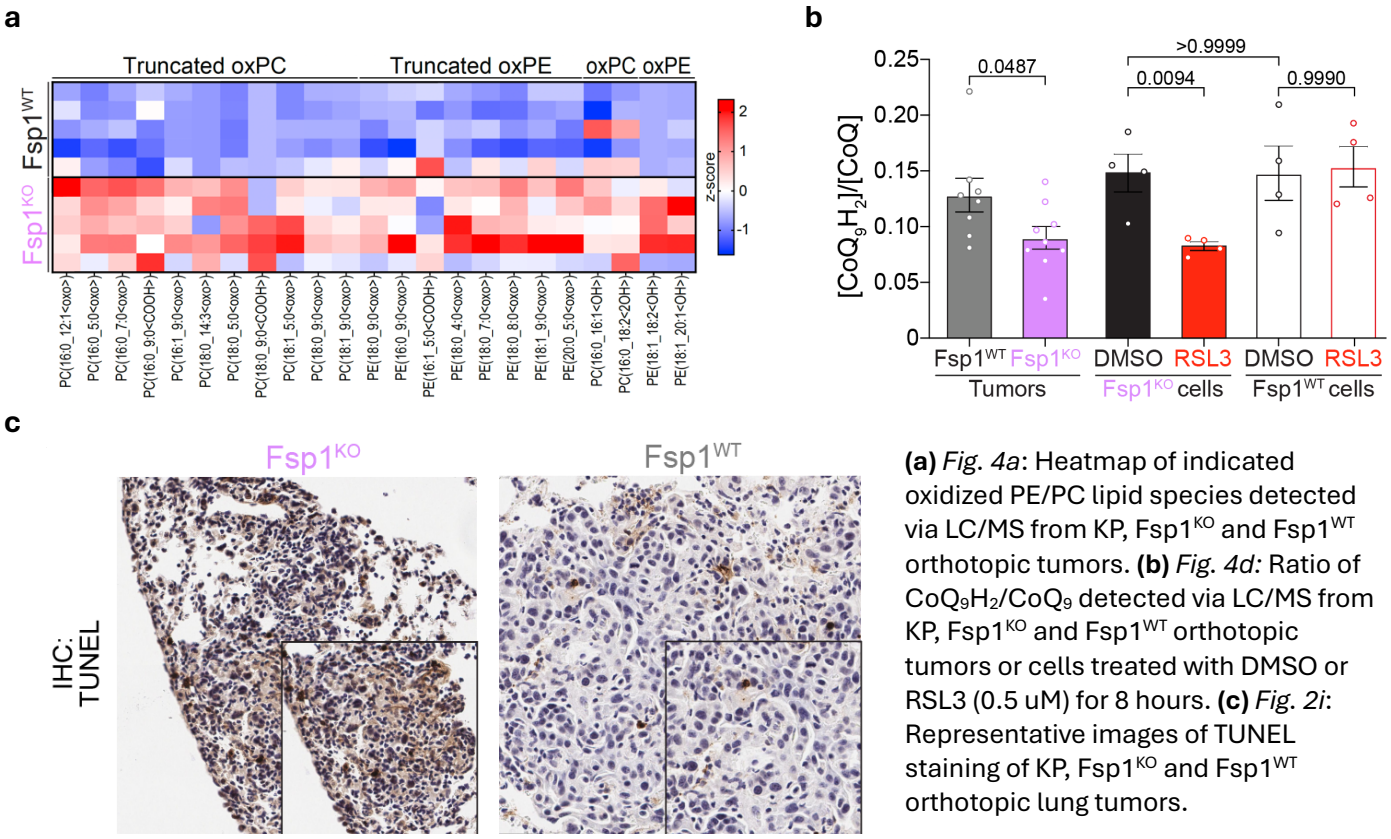
We thank the reviewer for highlighting our surprising discovery that both Gpx4 and Fsp1 independently contribute to malignant progression. We hypothesize that the most likely mechanism underlying tumor suppression upon Gpx4 and, more interestingly, Fsp1 loss is cell death via elevated lipid peroxidation and ferroptosis. To initially test this hypothesis, we performed a series of experiments in which the suppression of lipid peroxidation, whether by either Liproxstatin-1 (LIP1) treatment (Fig. 4j-l, Ext. Data Fig. 6m-o), high dietary Vitamin E (Fig. 4g-i), or *Acsl4* deletion (Fig. 4f, Ext. Data Fig. 6i-k), resulted in restoration of $Fsp1^{KO}$ tumor growth. These studies suggest that Fsp1's pro-tumorigenic effect is indeed mediated by lipid peroxidation, and it primarily functions to allow tumor cells to avoid ferroptotic cell death.

In the revised manuscript, we performed additional studies including epilipidomic analyses to quantify the levels of oxidized phosphatidylethanolamine (PE) and phosphatidylcholine (PC) species, in $Fsp1^{WT}$ and $Fsp1^{KO}$ tumors and found that $Fsp1^{KO}$ tumors had a higher abundance of not only oxidized PEs and PCs but also truncated forms of these PUFA-containing phospholipids (panel a below, Fig. 4a), which arise from unrepaired phospholipid peroxidation (see our recent Expert Recommendation, PMID: 40204928). In fact, several groups have recently highlighted the importance of truncated PE and PC species in the execution of membrane rupture and ferroptotic cell death (PMID: 38297129, 36870109, 29989809). Therefore, these results provide strong evidence that $Fsp1^{KO}$ tumors not only accumulate oxidized lipids that are susceptible to unchecked lipid peroxidation but are also distinctly poised to undergo ferroptosis, thereby leading to tumorigenic suppression. Lastly, an earlier study by the Vanden Berghe and Kagan labs showed that unrestrained phospholipid peroxidation unequivocally distinguishes ferroptosis from other cell death modes including pyroptosis, necroptosis, etc. (PMID: 33110056).

To address the question of possible alternative mechanism by which Fsp1 is pro-tumorigenic, we explored Fsp1's known function as an oxidoreductase for coenzyme Q, a potent lipid antioxidant. We used a newly developed, highly quantitative method to measure levels of reduced and oxidized CoQ_9 in murine $Fsp1^{WT}$ and $Fsp1^{KO}$ tumors *in vivo* as well as genotypically-matched cells *in vitro* (panel b below, Fig. 4d). Intriguingly, we observed that $Fsp1^{KO}$ tumors had a decreased ratio of reduced to oxidized CoQ_9, reflecting decreased cellular buffering capacity against lipid ROS, that was not seen *in vitro* at baseline (panel b below). Moreover, we found that RSL3 treatment of $Fsp1^{KO}$ cells *in vitro* mimicked the changes in reduced:oxidized CoQ_9 observed in tumors *in vivo.*

Additionally, as the reviewer points out, we did not observe differences in tumor proliferation or apoptosis with Fsp1 or Gpx4 deletion (Ext. Fig. 3d, e), which, in context with these data, further points to cell death driven by ferroptosis as the likely mechanism underlying decreased tumor burden. To directly show that Fsp1 loss triggers cell death *in vivo*, we performed TUNEL, a general marker of cell death including ferroptosis (PMID: 40204928, 35922516), staining in tumors. We observed a marked increase in TUNEL staining in $Fsp1^{KO}$ tumors when compared to $FSP1^{WT}$ controls (panel c below, Fig. 2i).

Our studies now demonstrate that $Fsp1^{KO}$ tumors: 1) Are not dying by apoptosis or display decreased proliferation; 2) Have increased levels of oxidized/truncated PE/PC lipids; 3) Have decreased reduced:oxidized CoQ_9; and 4) Are undergoing cell death as determined by *in situ* TUNEL staining. These results strongly support the notion that Fsp1 is essential for malignant progression of cancer by serving as a primary defense against ferroptosis. Hence, FSP1 loss triggers ferroptosis and robustly restricts tumorigenesis *in vivo*.



(a) *Fig. 4a*: Heatmap of indicated oxidized PE/PC lipid species detected via LC/MS from KP, Fsp1^{KO} and Fsp1^{WT} orthotopic tumors. **(b)** *Fig. 4d:* Ratio of CoQ_9H_2/CoQ_9 detected via LC/MS from KP, Fsp1^{KO} and Fsp1^{WT} orthotopic tumors or cells treated with DMSO or RSL3 (0.5 uM) for 8 hours. **(c)** *Fig. 2i*: Representative images of TUNEL staining of KP, Fsp1^{KO} and Fsp1^{WT} orthotopic lung tumors.

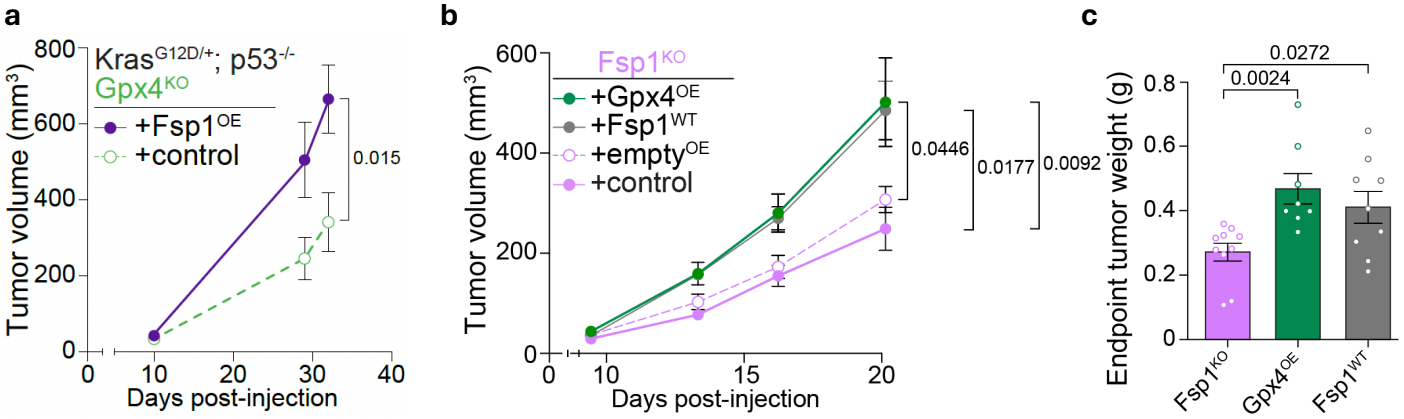
Altogether, these additional studies performed in response to the reviewer's questions provide further evidence that *in vivo* tumors experience higher levels of lipid peroxidation, and that both key ferroptosis surveillance systems are required to buffer cellular stress occurring during tumor progression. Upon the genetic loss or inhibition of either Fsp1 or Gpx4, this buffering capacity is diminished, resulting in in increased propensity to undergo ferroptotic cell death. This model would explain the suppression in tumorigenesis consistently observed *in vivo* with Fsp1 loss despite intact Gpx4 as detailed later (see response to comment #3).

3) FSP1 is critical for tumorigenesis independent of GPX4; however, in an in vitro setting, FSP1 seems to be dispensable. Additional experiments suggest that FSP1 exerts its pro-tumorigenic effects in a cell-autonomous manner. What could be the molecular mechanism underlying these intriguing findings? Why is GPX4 unable to compensate for the loss of FSP1 in vivo? Data presented in Figure 2d appear to indicate a growth advantage for tumors with high FSP1 expression. What are the functional differences between GPX4 and FSP1 in vivo concerning tumor progression? Does the overexpression of GPX4 in an FSP1-deficient condition reverse tumorigenesis? Conversely, what effect does overexpressing FSP1 in a GPX4-deficient condition have?

We share the reviewer's interest in further understanding the molecular mechanisms underlying the differential requirement for Fsp1 *in vivo* but not *in vitro*, as well as functional differences between Gpx4 and Fsp1 in regulating tumorigenesis *in vivo*. We performed the insightful experiments suggested by the reviewer to assess whether overexpression of Gpx4 or Fsp1 would rescue growth of tumors with loss of Fsp1 or Gpx4, respectively. We found that ectopic overexpression of Fsp1 in Gpx4^{KO} xenograft tumors significantly increased tumor growth (panel a below, Fig. 4b, Ext. Data Fig. 6a). Since it is well-established that Gpx4 loss, either *in vivo* or *in vitro*, triggers ferroptosis due to overwhelming lipid peroxidation, this data suggests that Fsp1 constitutes a primary backup system for suppressing lipid peroxidation to sustain tumor growth despite Gpx4 loss. Similarly, in the converse experiment, where we overexpressed Gpx4 in Fsp1^{KO} xenograft tumors, we observed a full rescue of tumor growth to nearly the same extent as Fsp1 reconstitution (panels b, c below, Fig. 4c, Ext. Data Fig. 6b-d).

Our findings from these additional *in vivo* studies, strongly implicate that tumors require extensive buffering capacity against lipid peroxidation and that while Gpx4 and Fsp1 act through distinct pathways, namely GSH and $CoQH_2$, each are able to compensate for the loss of the other. However, why tumors do not appear to upregulate the other anti-ferroptotic arm when one is lost, at least in this context, remains an open question that is undoubtedly interesting but at this time beyond the scope of our work.

Finally, as detailed in response to comment #1, our analyses found that $Fsp1^{KO}$ tumors had a decreased ratio of reduced to oxidized CoQ_9 which was also seen with $Fsp1^{KO}$ cells treated with RSL3 (panel b above, Fig. 4d). This was not observed with only Fsp1 genetic deletion or only RSL3-treatment *in vitro*, which suggests that Fsp1's ferroptosis-suppressing effect is most critical in a challenging microenvironment; in other words, since the metabolic demands of tumorigenesis *in vivo* inadvertently leads to generation of lipid peroxides, amongst other ROS, tumors *in vivo* experience a greater need for ferroptosis buffering capacity, which likely explains why Fsp1 is uniquely required *in vivo* but not *in vitro* unless the balance is shifted toward higher lipid peroxidation or contexts where Gpx4 is unable to buffer (i.e., $Gpx4^{KO}$, RSL3-treatment).



(a) *Fig4b*: Longitudinal growth of KP, $Gpx4^{KO} \pm Fsp1^{OE}$ subcutaneous (subQ) xenograft tumors transplanted in male C57BL/6J mice. (control, n=9, Fsp1OE, n=8). **(b)** *Fig. 4c*: Longitudinal growth of KP, $Fsp1^{KO} \pm Gpx4^{OE}$ or $Fsp1^{WT}$ subcutaneous (subQ) xenograft tumors transplanted in female C57BL/6J mice. (control, n=10; empty^{OE}; n=9, $Fsp1^{WT}$ n=9; $Gpx4^{OE}$, n=8). **(c)** *Ext. Data Fig. 6d:* Endpoint tumor weights from (b).

<u>Minor points</u>

Extended Data 3i and 3j are not referred in the text. Extended Data 4a is not referred in the text.

We thank the reviewer for noting this oversight and have revised the manuscript text to ensure all figure panels are referred to in the text.

Referee #2 (Remarks to the Author):
This is a manuscript by Wu et al where they have demonstrated targeting FSP1 as a therapeutic strategy in KRAS-driven lung cancer. By CRISPR-Cas9 gene editing, they noted that 1) Fsp1 deletion phenocopies Gpx4 KO in KP LUAD GEMM, 2) Fsp1 loss is sufficient to induce ferroptosis and reduce tumor burden even in the presence of Gpx4, 3) ferroptosis inhibition-genetically, pharmacologically, and dietarily- restores KP lung tumor growth, and 4) icFSP1 effectively inhibits tumor growth in vivo. The authors found FSP1 is an independent target for lung tumors and FSP1inhibition as a novel therapeutic strategy to improve prognosis of the KRAS-driven lung cancer patients. in lung cancer patients. However, given the aims and scope of the journal (suitable for wider public), the manuscript would fit more appropriately into cancer-specific journals (e.g., Nature Cancer).

We appreciate the reviewer's thoughtful point-by-point summary of the major conclusions from our studies.

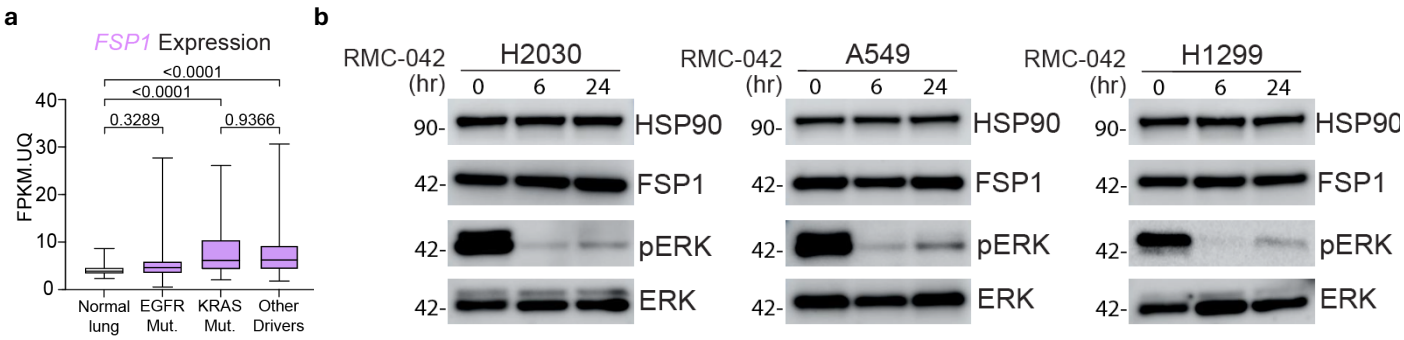
1) More mechanistic study: authors claim that Fsp1 loss reduces KRAS-driven lung tumor growth, and FSP1 levels are associated with patient outcome. It would be important to investigate 1) how oncogenic KRAS mutation can induce FSP1 expression, 2) if not, how KRAS mutation can create the environment where FSP1 is important for KRAS cancer growth. Also, it has been well known that concurrent mutations of KRAS and KEAP1 or KRAS and LKB1 increase oxidative stress more so than KP oncogenotype. I am wondering if the role of FSP1 (or GPX4) is more critical for molecular subtypes of KRAS-driven lung tumors.

2) It would be worth checking if it is KRAS-dependent or more general phenomenon by comparing to other lung cancer models with different driver mutations.

3) While KP LUAD GEMM is a wonderful tool to study tumorigenesis and tumor aggressiveness, it cannot give us information regarding the variants of KRAS mutations. In other words, it is unclear whether it is a KRAS G12D specific phenotype or other KRAS mutant-lung tumors would show the similar results. KRAS mutant PDXes might be good for the validation study.

Since the first three comments share a common theme and our responses are based on related data, we will address them together. We thank the reviewer for bringing up these astute points, and we agree that further delineation of whether certain molecular subtypes of lung cancer are more sensitive to FSP1 perturbation is important for the translational potential of these studies.

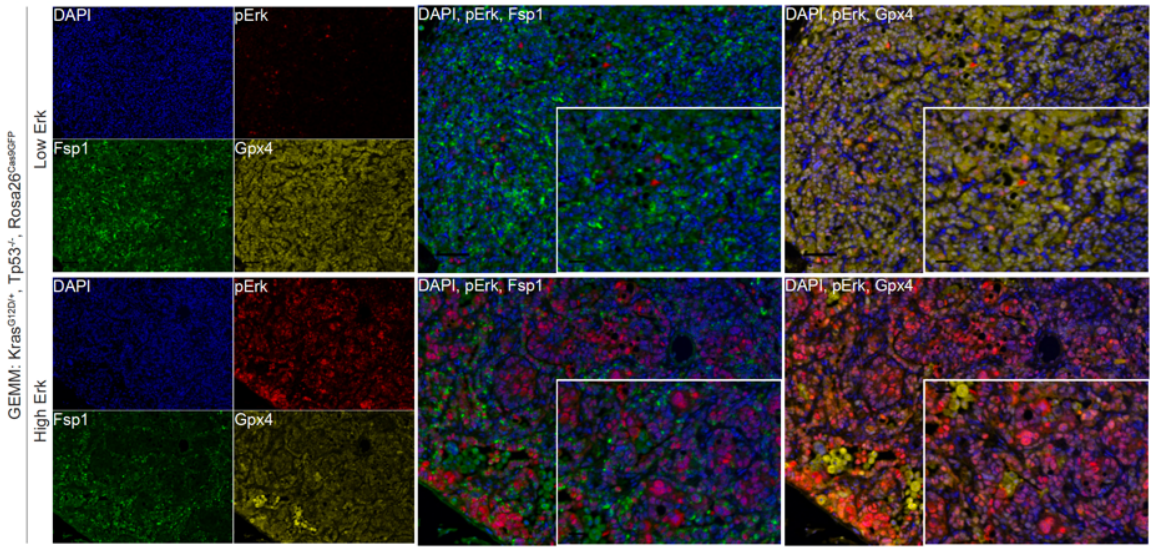
First, we recognize that several groups have recently linked FSP1 expression to KRAS signaling, specifically through NRF2-mediated transcription in human and mouse cells (PMID: 35459868, 36443441). However, specifically in the context of human LUAD, gene expression data from patient tumors from The Cancer Genome Atlas (TCGA) suggest that there is increased *FSP1* expression in lung tumors relative to normal lung irrespective of oncogenic driver mutations (i.e., *KRAS* and *EGFR)*. Although we do see that *FSP1* expression is more robustly increased in *KRAS*-mutant tumors than *EGFR*-mutant tumors, LUAD tumors without *EGFR* or *KRAS* mutations also have higher expression of *FSP1* to the same extent as *KRAS*-mutant tumors (panel a below, Ext. Data Fig. 2b). To assess the relationship between KRAS activation, and subsequent MAPK/ERK signaling, with FSP1 expression, we treated multiple human LUAD cell lines with the pan-RAS inhibitor, RMC-042, and found that FSP1 expression was unchanged despite modulation of ERK activation (panel b below, Ext. Data Fig. 2d). Based on these collective observations, we refrain from speculating that FSP1 expression is specifically or exclusively driven by KRAS.



(a) *Ext. Data Fig. 2b:* FSP1 (AIFM2) expression of primary LUAD tumors from TCGA, separated by oncogenic driver mutation (normal lung n=59, EGFR n=71, KRAS n=135, Other n=307). (**b**) *Ext. Data Fig. 2d*: Western Blot of KP human cells treated with 50nM RMC-042 for indicated time periods.

Furthermore, it is well known that in the KP GEMM there is chromosomal amplification of mutant *Kras* that leads to increased pErk signaling and enables progression from adenoma to adenocarcinoma. Therefore, we asked whether high pErk signaling in tumors correlates with changes in Gpx4 or Fsp1 expression. We performed multi-IF staining of KP tumors (panel c below, Ext. Data Fig. 2e) but failed to detect differences in Fsp1 protein levels in tumors with either high or low pERK, again suggesting that in the context of LUAD tumors, Fsp1 expression might not be directly dependent on Kras-induced Erk signaling.

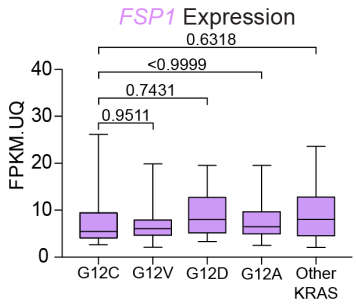
c



(c) *Ext. Data Fig. 2e:* Representative multi-IF images of KP tumors using indicated antibodies. Panels 10X, insets 20X.

Next, we assessed whether there are differences in FSP1 expression depending on the KRAS variant based on TCGA LUAD data. We did not observe a significant difference in FSP1 expression across different oncogenic *KRAS* mutations (panel d below, Ext. Data Fig. 2c).

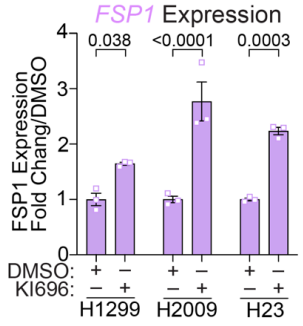
d



(d) *Ext. Data Fig. 2c:* FSP1 (AIFM2) expression of *KRAS*-mutant primary LUAD tumors from TCGA, separated by the *KRAS* mutation (G12C n=51, G12V n=32, G12D n=17, G12A n=16, Other KRAS n=20).

We next examined whether commonly occurring co-mutations impact FSP1 expression. First, we examined the relationship between NRF2 (activated by *KEAP1* loss-of-function mutations) in regulation of FSP1. Our studies in human LUAD cells showed that pharmacological KEAP1 inhibition *in vitro* did lead to a significant induction in *FSP1* gene expression (panel e below, Ext. Data Fig. 2g). Similarly, analysis of gene expression data from patient tumors from The Cancer Genome Atlas (TCGA) suggested that indeed *KEAP1* and *STK11* (LKB1) mutations, correlate with markedly increased *FSP1* expression in tumors (panel f below, Ext. Data Fig. 2f). These data, along with the existing literature, suggest that NRF2 activation does promote FSP1 expression, although the exact mechanism of regulation is not fully understood and outside the scope of our present studies.

e



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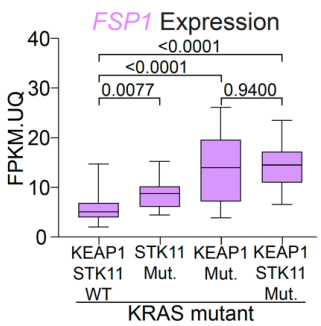


(e) *Ext. Data Fig. 2g:* qPCR of FSP1 expression from indicated human cell lines treated with an NRF2 inhibitor, KI696, for 72 hours. **(f)** *Ext. Data Fig. 2f:* FSP1 (AIFM2) expression of *KRAS*-mutant primary LUAD tumors from TCGA, separated by the co-mutation status of the tumor (KEAP1/STK11 WT n=88, STK11 mutant n=20, KEAP1 mutant n=16, KEAP1/STK11 mutant n=11).

To more systematically assess whether FSP1 loss is sufficient to decrease tumor growth irrespective of specific *KRAS* mutation, co-mutation with *KEAP1* or *STK11*, *EGFR* mutation, or tissue lineage, we performed a series of additional experiments utilizing a number of murine and human cell lines with varied mutations and lineages. We consistently found that FSP1 deletion decreased tumor growth *in vivo* (panels h-n below, Fig. 3a-g), irrespective of genotype, but had no effect on cell viability *in vitro* (Ext. Data Figs. 5a-g). These data strongly corroborate our initial observation of restricted tumor growth upon FSP1^{KO} and suggest that this phenotype is not specific to solely *KRAS*^{G12D}-mutant tumors. In fact, our studies demonstrate that, in general, tumors of different oncogenic drivers and co-mutations, are all sensitive to FSP1 deletion. Our PDAC data also suggests that this dependency may be generalizable to other tissues, but this would need to be further validated in different tumor types and could depend on the tissue lineage and tumor microenvironment. For simplicity, all utilized cell lines, and their genotypic information are summarized in a table (panel g below, Ext. Data Fig. 7l). Accompanying data from *in vitro* assays demonstrating increased sensitivity to RSL3 following FSP1^{KO} are not included below but are available in Ext. Data Fig. 5.

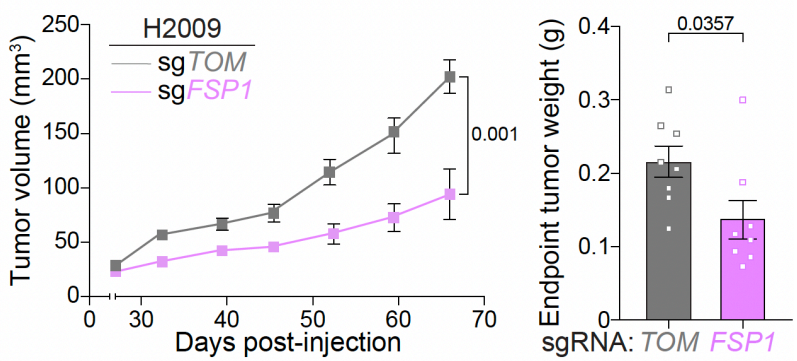
g

Cell Line	Oncogenic driver	Co-mutations	Tumor type
1234	Kras G12D	Tp53$^{-/-}$	mouse LUAD
H2009	KRAS G12A	TP53^{R273L}	Human LUAD
(PDX) LX465	KRAS G12D	TP53 L35fs8*	Human LUAD
H1299	NRAS Q61L	TP53^{del}	Human LUAD
PC9	EGFR Exon19Del	TP53^{R248Q}	Human LUAD
H1975	EGFR L858R/ T790M	TP53^{R273H} PI3KCA^{G118D}	Human LUAD
KPC7	Kras G12D	Tp53$^{-/-}$	mouse PDAC
A549	KRAS G12S	KEAP1^{G333C} STK11^{Q37C*}	Human LUAD
16645	Kras G12D	Stk11$^{-/-}$	mouse LUAD

(g) *Ext. Data Fig. 7l:* Table of respective mutations and tissue lineage from the cell lines used in this work.

H2009: Kras^{G12A}/TP53^{R273L} (Fig. 3a, Ext. Fig 5a)

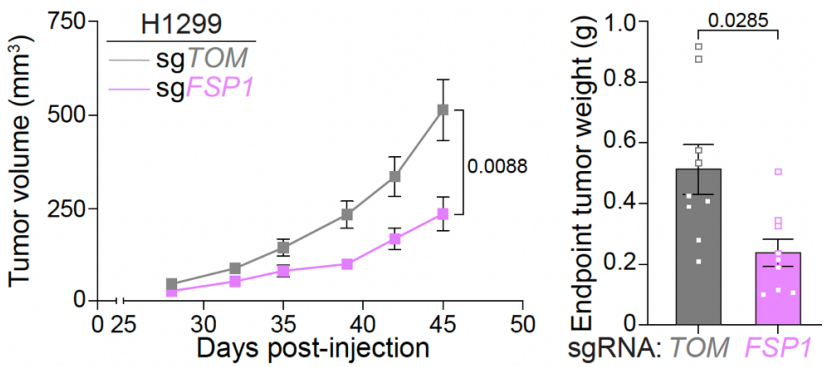
h



(h) Longitudinal growth (left) and endpoint tumor weights (right) from H2009 cells with CRISPR/Cas9-mediated knockout of FSP1 or non-targeting control, transplanted as subcutaneous (subQ) xenograft tumors into NSG mice. sgFSP1 (n=8) or control (n=8).

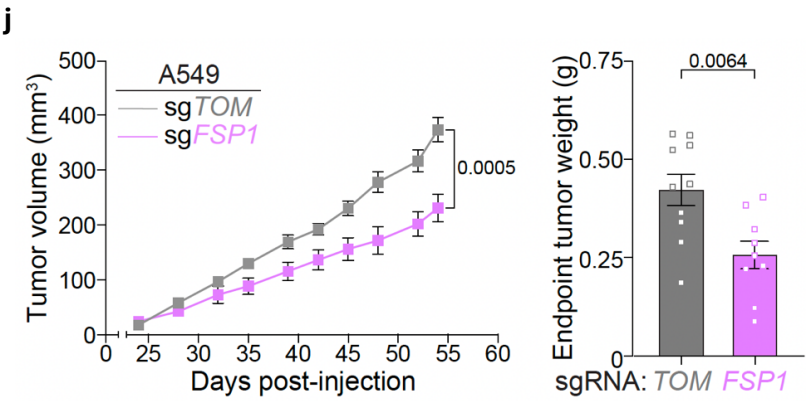
H1299: NRAS^{Q61K}/TP53^{del} (Fig. 3b, Ext. Fig 5)

i



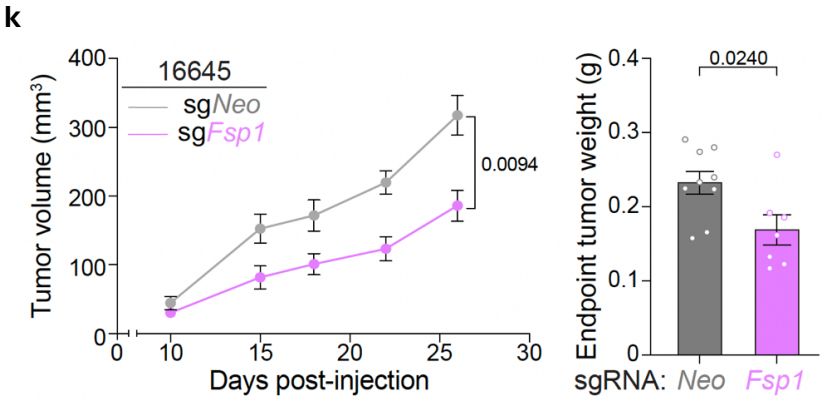
(i) As in h, but with H1299 tumors. FSP1 (n=9) or control (n=9).

A549: KRAS^{G12S}/KEAP1^{G333C}/STK11^{Q37C*} (Fig. 3e, Ext. Fig. 6e)



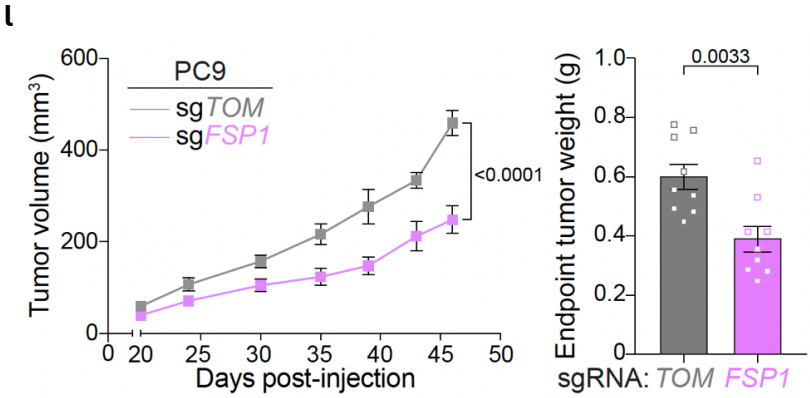
(j) As in h, but with A549 tumors. FSP1 (n=10) or control (n=9).

16645: Kras$^{G12D/+}$/Stk11$^{-/-}$ (Fig. 3f, Ext. Fig. 6f)



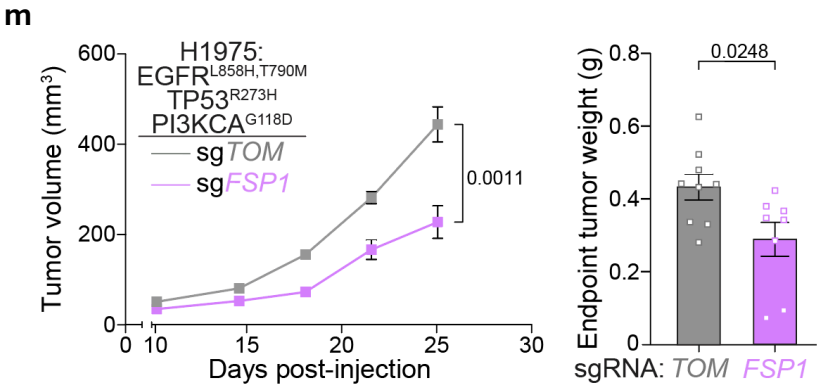
(k) As in h, but with 16645 tumors. Fsp1 (n=7) or control (n=9).

PC9: EGFR^{ex19del}/TP53^{R248Q} (Fig. 3c, Ext. Fig. 5c)



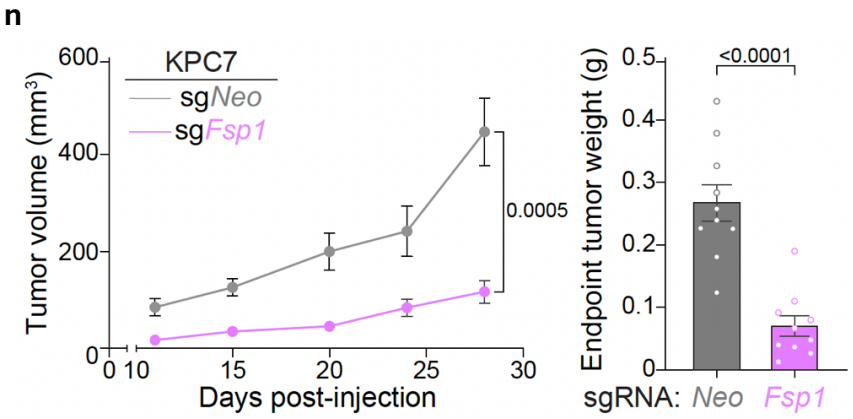
(l) As in h, but with PC9 tumors. FSP1 (n=9) or control (n=9).

H1975: EGFR$^{L858R/T790M}$/TP53^{R273H}/PI3KCA^{G118D} (Fig. 3d, Ext. Fig. 5d)



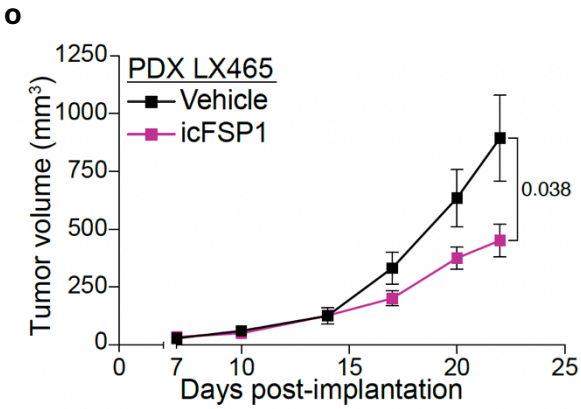
(m) As in h, but with H1299 tumors. FSP1 (n=8) or control (n=9).

KPC7: Kras$^{G12D/+}$Tp53$^{-/-}$ (PDAC) (Fig. 3g, Ext. Fig 5g)



(n) As in h, but with KPC7 tumors. FSP1 (n=10) or control (n=10).

Lastly, we utilized a KRAS^{G12D}, TP53-mutant PDX model (LX465) to further assess the therapeutic potential of FSP1 inhibition using icFSP1 (panel o below, Fig. 5d). Excitingly, we found that icFSP1 treatment significantly slowed PDX tumor growth, further bolstering the translational potential of targeting FSP1 in LUAD patients.



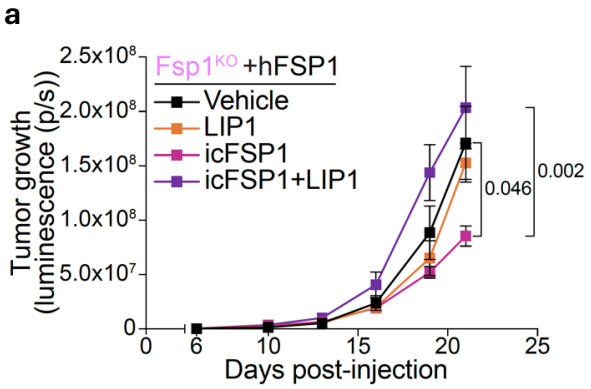
(o) *Fig. 5d:* Longitudinal growth of patient-derived xenograft (PDX) tumors treated with icFSP1 (50 mg/kg BID) (n=10) or Vehicle (n=10) in female NSG mice.

4) It has been reported that pathologically activated neutrophils, known as polymorphonuclear (PMN) myeloid-derived suppressor cells (MDSCs), undergo spontaneous ferroptosis, which contributes to immune suppression in cancer (PMID: 36385526). Given the important roles of ferroptosis both in tumor and tumor microenvironment, it would be important to investigate whether systemic FSP inhibition impacts tumor growth and how FSP1 inhibition in the TME affects FSP1 inhibition-mediated tumor reduction.

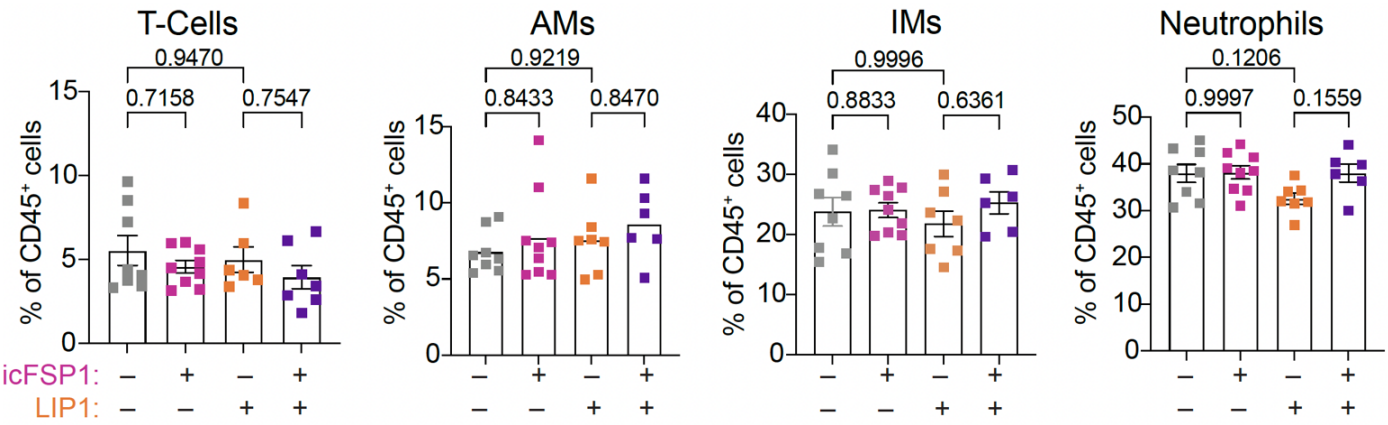
We thank the reviewer for bringing up this point, and we agree with the reviewer that understanding the role of ferroptosis in the tumor microenvironment is an important area of investigation that our newly developed mouse models can contribute to. Uncovering how FSP1 systemic inhibition may impact specific immune populations is a crucial step to the advancement of therapeutic approaches targeting ferroptosis in patients. However, the lack of pharmacological FSP1 inhibitors with sufficient metabolic stability and appropriate pharmacokinetic properties in animal models makes it challenging to directly dissect the degree to which FSP1 inhibition in the TME affects tumor reduction. Of note, the only multi-species inhibitor viFSP1 (PMID: 37957306) is not suitable for *in vivo* use. icFSP1 is the only FSP1 inhibitor that has been shown to have efficacy *in vivo* but is specific to the human ortholog of FSP1 (PMID: 37380771). To overcome this technical hurdle, the icFSP1 treatment experiments carried out in this manuscript utilized orthotopic transplantation of Fsp1^{KO} LUAD murine cells with re-expression of the human isoform of FSP1. In these experiments, we are assessing the therapeutic benefit of tumor-specific induction of ferroptosis via FSP1 inhibition.

To more directly answer the question of the effect of tumor-specific FSP1 inhibition on the TME, we performed multi-color FACS profiling of the innate myeloid and T-cell populations in tumor-bearing lungs from animals treated with icFSP1. Importantly, in this new experiment, we again observed that icFSP1 significantly suppressed the growth of tumors and additionally found that LIP1 supplementation was able to fully rescue the tumor growth in icFSP1-treated animals (panel a below, Fig. 5b). Based on our FACS analyses of these tumors, we found that there were no significant changes in the proportion of T-cells or macrophages (alveolar or interstitial) across any of the treatment groups (panel b below, Ext. Data Fig. 7j). Furthermore, per the reviewer's suggestion, we profiled PMN MDSCs which we designated below as Neutrophils. Similar to the other immune cell populations

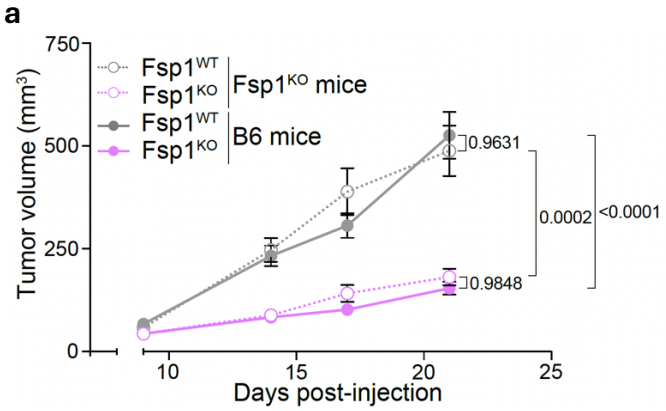
investigated, we did not observe any significant changes in this population between treatment groups, albeit with a small but insignificant decrease in the LIP1 treated animals (panel b panel, Ext. Data Fig. 7j). Taken together, these data suggest that icFSP1 treatment and tumor-specific inhibition of FSP1 does not induce major changes to the lung immune TME and implies that the decrease in tumor burden in icFSP1-treated animals is primarily due to cell-autonomous effects. However, additional more comprehensive immune profiling studies, which are outside the scope of our work, will need to be performed to fully examine whether changes in distinct transcriptional subsets of T cells (e.g. Th1, Treg, Th17) or innate immune cells (e.g. dendritic cells), are impacted by tumor-specific inhibition of FSP1.

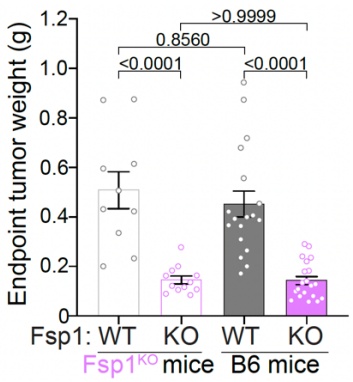


(a) *Fig. 5b:* Tumor bioluminescence signal of C57BL/6J mice orthotopically transplanted with hFsp1^{WT} cells and treated with either Vehicle (n=8), LIP1 (10mg/kg daily) (n=7), icFSP1 (50 mg/kg BID) (n=8), or icFSP1+LIP1 (n=8).
(b) *Ext. Data Fig. 7j:* Percent of T-cells, alveolar or interstitial macrophages, and neutrophils of total CD45+ cells from tumor bearing lungs treated with the indicated compounds for two weeks (Veh, n=7, LIP1, n=7, icFSP1, n=9, icFSP1+LIP1, n=6).



Next, we assessed whether Fsp1 loss in the TME generally has an impact on the growth of Fsp1^{WT} or Fsp1^{KO} tumors. We utilized full-body Fsp1 knockout mice (C57BL6/J) generated by the Conrad group (PMID: 35922516) and performed transplantation studies to investigate whether there was a differential impact on tumor growth compared to age- and gender-matched B6 Fsp1^{WT} (C57BL6/J) hosts. Consistent with all our prior tumor growth *in vivo* studies, we observed decreased tumor volume and endpoint tumor weight of Fsp1^{KO} tumors in both WT and Fsp1 knockout host mice (panel a below, Ext. Data Fig. 4i). We recognize that further work is required to assess the effect of systemic FSP1 inhibition on both tumors and the TME once newer inhibitors targeting murine Fsp1 with sufficient PK/PD properties are available. Regardless, these data do indicate that the effects of future therapeutic approaches targeting FSP1 will most likely be driven by tumor-intrinsic effects via induction of tumor cell ferroptosis.





(a) *Ext. Data fig. 4i* Longitudinal growth and endpoint tumor weight of Fsp1^{KO} or Fsp1^{WT} subQ xenograft tumors transplanted in C57BL/6J WT (KO, male n=10, female n=10, WT, male n=8, female n=10) or C57BL/6J Fsp1 KO mice (males and females, KO, n=12, WT, n=10).

Referee #3 (Remarks to the Author):

Induction of ferroptosis through the inhibition of cellular defense systems (i.e., GPX4 and FSP1) has been proposed as a strategy to kill therapy-resistant cancer cells. However, whether this is a viable therapeutic approach remains an open question.

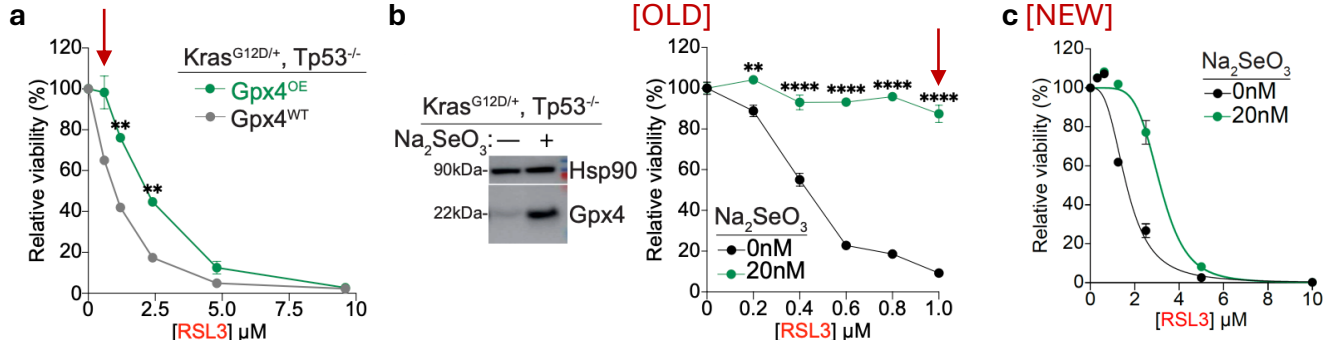
In the current manuscript, the authors explore the potential to target GPX4 and FSP1 in lung cancer. Genetic depletion of GPX4 or FSP1 are sufficient to suppress lung tumor growth. The effect of GPX4 depletion was not surprising given the numerous studies showing its essentiality in many types of cancer, though the findings in this immunocompetent GEM model are a nice addition to these reports. The surprising finding was that FSP1 depletion strongly suppressed tumor growth. This finding is in contrast to in vitro data, where knockout of FSP1 or inhibition of FSP1 does not trigger ferroptosis on their own. Similar findings are observed with both the GEM and a complementary orthotopic xenograft model. The authors further show that FSP1 expression increases with the stage of lung cancer and FSP1 expression correlates with poor patient prognosis. Loss of FSP1 results in increased H&E staining, indicative of lipid peroxidation. The H&E staining and tumor suppression are reversed by treatment with known ferroptosis inhibitors such as Lip1 and vitamin E. Finally, the authors find that the FSP1 targeting molecule icFSP1 increases the survival of mice in an orthotopic lung tumor model.

This is a very nice and important study that is the first to demonstrate the benefit of single agent targeting of FSP1 in cancer, uncovering a ferroptosis vulnerability that is present in vivo but not in vitro. The study is carefully performed and complementary data from multiple models support the conclusions. The data presented address a key question in the field and the findings with these sophisticated mouse models are exciting and have important therapeutic implications for targeting FSP1 and ferroptosis in cancer. There are some comments that should be addressed.

We thank the reviewer for their complementary description of our work, its importance in the field, and its implications for the therapeutic benefit for lung cancer patients in targeting ferroptosis through FSP1 inhibition.

1) Overexpression of GPX4 had a mild effect on the resistance to RSL3-induced ferroptosis (extended fig 1f). In contrast, sodium selenite (Na2SeO3) addition completely blocked RSL3-induced ferroptosis (extended fig 1g). The difference is perhaps unexpected and seems to suggest that Na2SeO3 is protecting cells from ferroptosis through a mechanism independent of GPX4. Please explain further.

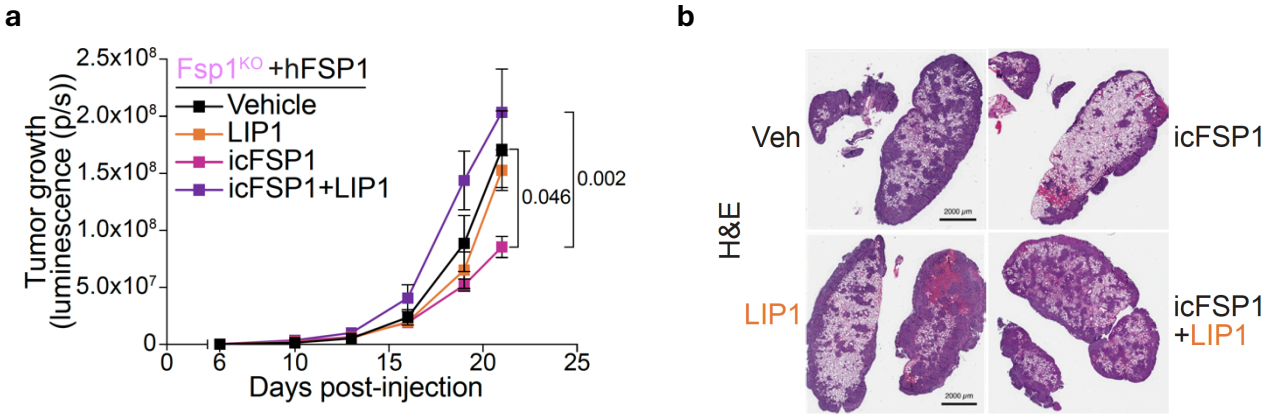
We appreciate the reviewer for pointing out this discrepancy in resistance to RSL3-induced ferroptosis between ectopic Gpx4-overexpression and Na_2SeO_3 supplementation. One notable caveat is that the concentrations of RSL3 used were different for these *in vitro* studies (up to 10uM in the former versus 1uM in the latter). Although Na_2SeO_3 may have appeared to more robustly protects cells from ferroptosis, there was actually no marked difference in viability at 1uM RSL3- with both Gpx4-overexpressing and Na_2SeO_3-supplemented KP cells at near 100% viability (red arrows, panels a and b). However, we acknowledge that the way the data was shown in the initial submission is confusing, so we have repeated the experiment to show that RSL3-resistance with Na_2SeO_3 supplementation closely mirrors that of Gpx4-overexpression (panel c, Ext. Data Fig.1g). Another important caveat in response to the reviewer's broader question about possible Gpx4-independent mechanisms by which Na_2SeO_3 confers ferroptosis resistance is that the level of Gpx4 overexpression *in vitro* is highly dependent on the amount of selenium in cell culture medium. Typically, only 10% FBS or FCS is used, which serves as the sole source of selenium, as recently highlighted in our Expert Recommendation article (PMID: 40204928). In other words, without additional Na_2SeO_3 supplementation, Gpx4 expression is limited by the selenium content of the serum, which may explain discrepancies in cell viability observed at similar RSL3 concentrations.



(a) Ext. Data Fig. 1f: CellTiter-Glo Luminescence viability assay of KP, Gpx4 WT or OE cells upon increasing concentrations of RSL3. **(b)** *Ext. Data Fig. 1g:* Western blot of KP LUAD cells treated with 20nM Na2SeO3 or DMSO. CellTiter-Glo Luminescence viability assay of KP LUAD cells treated with 20nM Na2SeO3 or DMSO with increasing RSL3 addition.

2) icFSP1 treatment extended the survival of the mice bearing orthotopic lung tumors. Did this treatment affect tumor size? Is this reversed by Lip1?

To address these astute questions, we repeated the same icFSP1 treatment experiment using the humanized hFSP1-expressing orthotopic lung tumor model described and used IVIS bioluminescence imaging to track the longitudinal growth of the lung tumors in response to the different treatments. In this newer iteration of the experiment, we included two additional treatment arms, LIP1 only and icFSP1+LIP1, to investigate whether we are able to rescue the growth of tumors treated with icFSP1. As mentioned earlier (see reviewer #2, comment #4) and in correlation with the survival data shown in the initial manuscript (also see response to comment #3, panels a and b), we observed that icFSP1 treatment led to significantly decreased tumor growth in comparison to Vehicle-treated animals (panels a and b below, Fig. 5b and Ext. Data Fig. 7i). Importantly, this therapeutic benefit was eliminated with concomitant LIP1 dosing. This suggests not only that icFSP1 decreases KP tumor growth, but that the effect of icFSP1 treatment in this model occurs via the induction of ferroptosis.

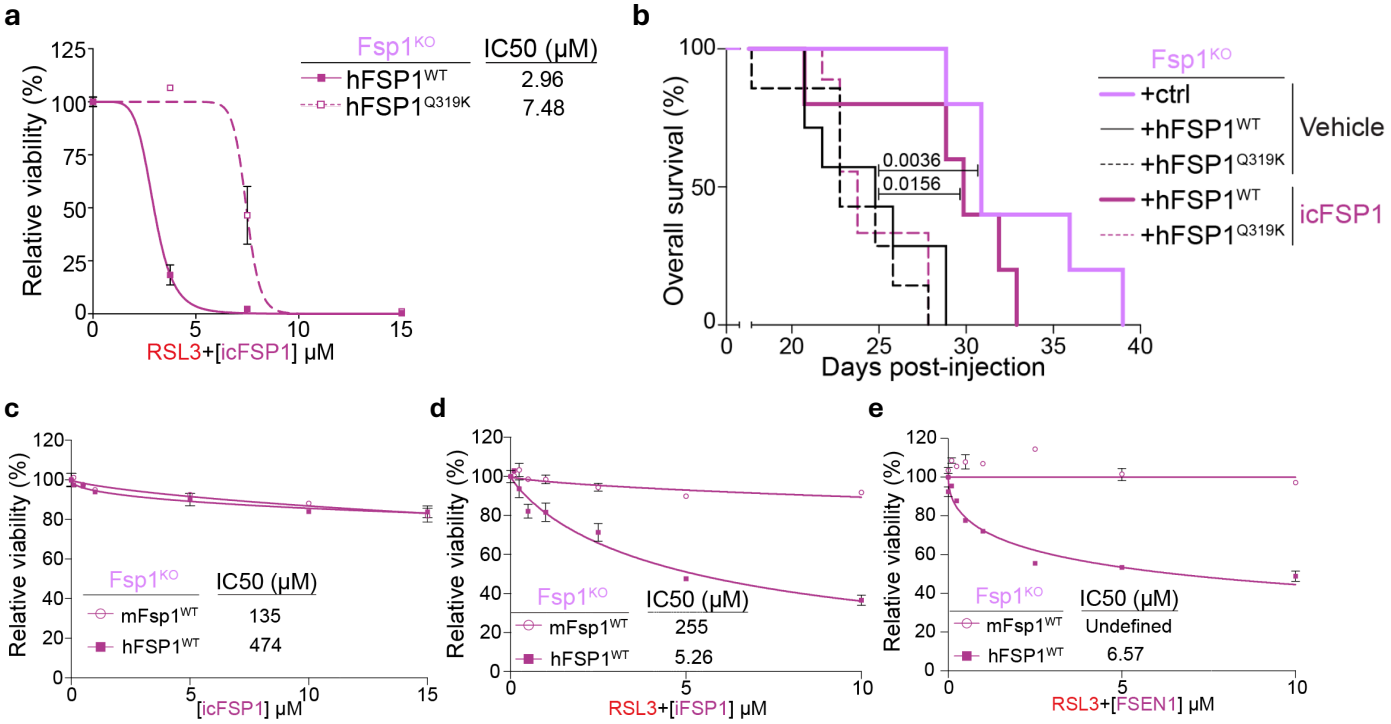


(a) *Fig. 5b:* Tumor bioluminescence signal of C57BL/6J mice orthotopically transplanted with hFsp1^{WT} cells and treated with either Vehicle (n=8), LIP1 (10mg/kg daily) (n=7), icFSP1 (50 mg/kg BID) (n=8), or icFSP1+LIP1 (n=8). **(b)** *Ext. Data Fig. 7i*: Representative H&E of KP, hFsp1WT orthotopic lung tumors from indicated treatment groups.

3) icFSP1 is a unique "inhibitor" in that it acts by triggering the phase separation of FSP1. In contrast to other FSP1 inhibitors and genetic deletion of FSP1, icFSP1 has been found to have some single agent activity in vitro (PMID: 37380771). This calls into question the selectivity of the molecule or whether it might be acting through a gain of toxicity rather than loss of FSP1 activity. Would you see a similar effect using a more traditional inhibitor of FSP1 activity?

Unfortunately, unlike icFSP1, more traditional inhibitors of FSP1 such as iFSP1 do not have efficacy *in vivo* and thus were unable to be used for our studies to demonstrate the therapeutic benefit of targeting FSP1 in tumors. However, we do agree with the reviewer's comment that icFSP1's unique mechanism and potential toxicity/off-target effects may confound the therapeutic benefit we have reported. In an effort to demonstrate that icFSP1 indeed exerts on-target FSP1 inhibition in tumor cells, we included an internal control within our icFSP1 treatment experiments with mice that harbor tumors expressing an inhibitor-resistant mutant (Q319K) of FSP1, which had been previously shown to prevent icFSP1-mediated phase separation and thus disrupt FSP1 function (PMID: 37380771) (panel a below, Ext. Data Fig. 7f). Accordingly, our studies showed that icFSP1 had no therapeutic effect on FSP1^{Q319K}-mutant tumors *in vivo* (panel b below, Fig. 4c), which reassured us that the benefit in overall survival seen with icFSP1 was most likely due to specific, selective activity against tumor cells expressing its target rather than general pharmacological toxicity of the drug. Furthermore, given that LIP1 was able to restore growth of icFSP1-treated tumors (see response above to reviewer point #2), we believe that icFSP1 specifically targets FSP1 in tumor cells to suppress its lipid peroxidation-inhibiting role and induce ferroptosis, which can be blunted with lipid antioxidants like LIP1. Lastly *in vitro*, icFSP1 did not have any appreciable single agent activity but rather required additional RSL3 to see differential cell viability, just as we have consistently observed with

Fsp1 genetic knockout (panel c below, Ext. Data Fig. 7e). While we could not test other FSP1 inhibitors *in vivo*, we did observe that iFSP1 and FSEN1 treatment in combination with RSL3 sensitized cells *in vitro* as seen with icFSP1 (panels d and e below, Ext. Fig. 7c and d).



(a) *Ext. Data Fig. 7f:* CellTiter-Glo Luminescence viability assay of WT or Q319K-mutant hFSP1-expressing cells upon RSL3 (0.2μM) plus increasing concentrations of icFSP1. **(b)** *Fig. 4c:* Overall survival of C57BL/6J mice with either $Fsp1^{KO}$ tumors (n=5), $hFSP1^{WT}$-expressing tumors, or $hFSP1^{Q319K}$-expressing tumors, treated with icFSP1 ($hFSP1^{WT}$, n=6; $hFSP1^{Q319K}$, n=7) or Vehicle ($hFSP1^{WT}$, n=7; $hFSP1^{Q319K}$, n=7). **(c)** *Ext. Data Fig. 7e:* CellTiter-Glo Luminescence viability assay of human or mouse expressing $Fsp1^{WT}$ cells upon increasing concentrations of icFSP1. **(d)** *Ext. Data Fig. 7c:* CellTiter-Glo Luminescence viability assay of human or mouse expressing $Fsp1^{WT}$ cells upon RSL3 (0.2μM) plus increasing concentrations of iFSP1. **(e)** *Ext. Data Fig. 7d:* CellTiter-Glo Luminescence viability assay of human or mouse expressing $Fsp1^{WT}$ cells upon RSL3 (0.2μM) plus increasing concentrations of FSEN1.

Minor comments

1) A statement is made regarding the importance of GPX4 in regulating tumor growth - “However, its importance in regulating tumor growth remains unknown.” (lines 92-92) This seems to be a bit of an overstatement given the number of studies exploring GPX4 in cancer. Some amount of refining of this statement would be helpful.

We thank the reviewer for pointing this out and have revised the statement accordingly.