nature portfolio

Peer Review File

USP35 promotes the growth of ER positive breast cancer by inhibiting ferroptosis via BRD4-SLC7A11 axis

Corresponding Author: Professor Haihua Gu

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

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The results presented by Cao et al are consistent with previously published findings on regulation of cellular ferroptosis machinery by USP35. Prior studies have shown stabilisation of ferroportin by USP35 in lung cancer models(1). DUBs have also been found to reduce ferroptosis by stabilising SLC7A112 (2). The novelty of this study relates to its focus on the epigenetic regulator BRD4 and the ER+ breast cancer disease context. Whilst recent reports have uncovered a roll for BRD4 in fatty acid metabolism and ferroptosis (3), the research field is still in its infancy. Cao et al present a well-conceived and interesting study which mechanistically defines a novel function of BRD4 in regulating ferroptosis in ER+ breast cancer models.

Major points
1. Measurement of lipid ROS level with BODIPY C11. In figures 1-3, flow cytometry analysis of lipid ROS show only minor shifts in signal. Quantification and statistics are performed on the percentage of positive cells, but the MFI values for fluorescence should also be shown. BODIPY C11 use often reveals significant shifts in signal intensity, especially when using ferroptosis inducers such as ML162. This is particularly apparent in Figure 3F, were RSL3-treated cells exhibit overlapping histograms with the vehicle condition.
2. Figure 3 – Is USP35 regulating of SLC7A11 directly by deubiquitination? Measuring SLC7A11 ubiquitination level or MG132 usage in these assays will reveal this.
3. Figure 5D – although the TCGA analysis shows a positive correlation between BRD4 and SLC7A11, USP35 control of BRD4 is not transcriptional. Consulting a proteogenomic dataset (e.g. CPTAC) to determine correlations between protein levels in primary breast tumours would be valuable here.
4. Figure 5 – Title “BRD4 inhibits ferroptosis” is not justified by the data within the figure. Although the BRD4 inhibitor JQ1 increases lipid ROS levels, an additional experiment showing sensitivity of BRD4 shrRNA cells to RSL3-mediated killing would strengthen this (perhaps a colony formation assay).
5. The authors state that USP35 is upregulated by estrogen signaling, however it would be good to determine how dependent the USP35-BRD4-SLC7A11 mechanism is on ER status and estrogen sensitivity. Does USP35 over-expression in an ER- cell line (e.g. MDA-MB-231) also render cells resistant to ferroptosis?

Minor points
1. Figure 2D – is the over-expression really just 15-fold higher than the control? It looks more.
2. Line 352 “players”? Needs clarification.
3. Figure 5D – Did the authors test TNBC tumours? Was the correlation still present?
4. Figure 7D – The photographs of mice contribute little to the data so should be removed.

Citations

1. Tang, Z. et al. Deubiquitinase USP35 modulates ferroptosis in lung cancer via targeting ferroportin. Clin. Transl. Med. 11, e390 (2021).
2. Liu, T., Jiang, L., Tavana, O. & Gu, W. The deubiquitylase OTUB1 mediates ferroptosis via stabilization of SLC7A11. Cancer Res. 79, 1913–1924 (2019).

3. Yang, M. et al. Bromodomain-containing protein 4 (BRD4) as an epigenetic regulator of fatty acid metabolism genes and ferroptosis. Cell Death Dis. 13, 1–17 (2022).

Reviewer #2

(Remarks to the Author)
The manuscript by Cao et al. report that USP35, commonly upregulated in ER+ breast cancers, contributes to tumour growth via the inhibition of ferroptosis. Knockdown of USP35 in ER+ breast cancer cell lines increased several markers of ferroptosis, whilst USP35 overexpression reduced ferroptosis-mediated cell death. The authors uncover a potential mechanism for this effect, with USP35 interacting with and deubiquitinating BRD4, which leads to further upregulation of the cysteine transporter SLC7A11. Interactions were uncovered between USP35-BRD4, USP35-SLCA11 and BRD4-SLC7A11 in cell lines to support this feedback loop. An ER+ breast cancer in vivo model provided further evidence for the role of USP35 in promoting tumour growth. This is a well conducted article that has used appropriate methodology to investigate an interesting and novel area of research that will provide valuable information for the scientific community.

Major points to address:
1) In Figure 1C, especially for ZR-75-1, levels of cell viability look very similar following apoptosis or necrosis inhibition despite presentation as statistically significant. It would informative to investigate markers of apoptosis or necrosis following USP35 knockdown to confirm that ferroptosis is the only cell death mechanism inhibited by USP35.

2) The authors state that USP35 inhibits ferroptosis independently of BRD4 in TNBCs at line 406. It would be worthwhile to investigate if USP35 and BRD4 interact in a similar matter in TNBC cells as in ER+ breast cancer cells. It would also be interesting to see if TCGA analysis could be conducted on TNBC samples to investigate the association of USP35/ferroptosis and BRD4/SLC7A11 in this cancer type. The authors should discuss any potential evidence/theories for why ferroptosis would be inhibited in different ways by USP35 in these cancer types.

3) Is there any existing evidence for inhibited ferroptosis in ER+ breast cancer? If so, this should be discussed. Is there any literature indicating why ferroptosis would be selectively targeted in ER+ breast cancers? More detail should be provided on the current usage and how the results from this paper support the use of ferroptosis inducers in the treatment of this disease.

Minor points to address:
• Authors should include n numbers for all experiments.
• Figure 1I - Pearson’s correlation coefficient or equivalent and p value should be included more clearly.
• MFI values should be included in FACS histogram plots – can be difficult to visualise small differences between peaks of different conditions.
• Figure 2A – scale bars should be included for cell viability – are they the same as Figure 2E? IF so, this should be consistent throughout the manuscript.
• Line 320: remove “to” in “to new protein synthesis”
• Line 384; Remove “our” between “previous findings”
• Figure 7G – labelled IHC plots incorrectly, 2x USP35+vehicle conditions.
• Could not find a reference to Figure 8 in the text.

Reviewer #3

(Remarks to the Author)
This manuscript by Cao et al. investigates the role of USP35 in estrogen receptor-positive (ER+) breast cancer, with a focus on ferroptosis, a form of cell death that has garnered significant attention in the research community. While anti-estrogen therapies have improved survival rates, around 30% of patients do not initially respond, highlighting the importance of exploring new treatments for resistant breast cancers. The authors demonstrate that USP35 inhibits ferroptosis by interacting with and stabilizing BRD4, a protein that regulates SLC7A11, which in turn prevents ferroptosis and promotes cancer cell growth. The findings suggest that targeting USP35, in combination with a ferroptosis inducer, may offer a promising therapeutic strategy for ER+ breast cancer resistant to endocrine therapies.

The authors have conducted a thorough literature review, referencing recent publications that support their findings in other cancer types. However, they missed citing Yan et al. 2023 in Nature Communications, which establishes a link between SLC7A11 and ferroptosis.

For the manuscript to be more convincing, the following concerns regarding rigor and reproducibility should be addressed:

Specific Comments on Rigor and Reproducibility:

1.Data Presentation: All bar graphs should include individual data points to allow for better assessment of sample size and distribution (currently, only Figure 7 displays individual points).

2.Statistical Analysis: Unpaired t-tests should not be used unless data follow a normal distribution. Given the potentially low

sample sizes, the Mann-Whitney test may be more appropriate.

3.Materials and Methods: The section lacks sufficient detail for reproducibility. Specific issues include:

Culture Conditions: Missing information on culture media, drug vehicles, cell seeding numbers, and experimental timelines.
References: Avoid vague statements like "previously described," which require readers to consult older references.
Reagent Details: The dilution used for BRD4 is not specified.
Immunoprecipitation (IP): Details such as protein quantities, method specifics, and IgG control references are absent.
Flow Cytometry (FACS): Unclear whether cells are only resuspended in PBS—no mention of FACS buffer or live/dead dyes to exclude non-viable cells.

4.IHC Scoring: A supplemental figure should be provided, showing examples of each scoring category (0/1/2/3) to help readers appreciate the differences.

5.TCGA Data: The datasets used for TCGA analysis should be clearly cited.

6.Experimental Replicates: Biological and technical replicates (n values) should be specified in the figure legends.

7.Correlation Data:

Figure 1, panel I, lacks the correlation coefficient, which is essential to assess the described negative correlation.
Figure 4, panel H, shows a discrepancy between the indicated sample size (n=18) and the number of dots present (only 11).
Figure 4, panel D, also lacks a correlation coefficient.

By addressing these issues, the manuscript will significantly improve in terms of scientific rigor and reproducibility, enhancing its overall impact.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have addressed my queries with their revisions and rebuttal.

- Paul T. Kennedy

Reviewer #3

(Remarks to the Author)
I would like to sincerely thank the authors for their thoughtful and thorough responses to my comments and concerns. Their detailed explanations and revisions have significantly enhanced the clarity and rigor of the manuscript. These improvements have strengthened the manuscript's impact and contributed meaningfully to the field.
However, I am still not convinced of the use of the T-test for analyzing the small sample size. Three points per condition seems relatively small to assess the normality of the data. This is my only remaining concern about this study.

Reviewer #1 (Remarks to the Author):

The results presented by Cao et al are consistent with previously published findings on regulation of cellular ferroptosis machinery by USP35. Prior studies have shown stabilisation of ferroportin by USP35 in lung cancer models(1). DUBs have also been found to reduce ferroptosis by stabilising SLC7A11 (2). The novelty of this study relates to its focus on the epigenetic regulator BRD4 and the ER+ breast cancer disease context. Whilst recent reports have uncovered a roll for BRD4 in fatty acid metabolism and ferroptosis (3), the research field is still in its infancy. Cao et al present a well-conceived and interesting study which mechanistically defines a novel function of BRD4 in regulating ferroptosis in ER+ breast cancer models.

Response: We thank reviewer 1 for the overall positive assessment of our study and all the constructive suggestions.

Major points

1. Measurement of lipid ROS level with BODIPY C11. In figures 1-3, flow cytometry analysis of lipid ROS show only minor shifts in signal. Quantification and statistics are performed on the percentage of positive cells, but the MFI values for fluorescence should also be shown. BODIPY C11 use often reveals significant shifts in signal intensity, especially when using ferroptosis inducers such as ML162. This is particularly apparent in Figure 3F, were RSL3-treated cells exhibit overlapping histograms with the vehicle condition.

Response: We presented the quantification and statistics on the percentage of positive cells according to many published papers (PMID: 38297130, 38168770, 36864172). In addition, we have expanded the abscissa of the flow cytometry figure, which has enabled the shifts to be seen more clearly. Furthermore, we presented all the MFI values for fluorescence in Figure S9.

2. Figure 3 – Is USP35 regulating of SLC7A11 directly by deubiquitination? Measuring SLC7A11 ubiquitination level or MG132 usage in these assays will reveal this.

Response: The reviewer raised a valid question. We examined the protein level of SLC7A11 in MCF-7 and ZR-75-1 cells treated with MG132 according to the reviewer's suggestion. With MG132 treatment, SLC7A11 protein levels were increased in con-sh cells, but the treatment was unable to rescue the decrease in SLC7A11 protein levels in USP35-sh cells, suggesting that USP35 does not deubiquitinate SLC7A11 directly. This new data is included in Figure S3 of the revised manuscript.

3. Figure 5D – although the TCGA analysis shows a positive correlation between BRD4 and SLC7A11, USP35 control of BRD4 is not transcriptional. Consulting a proteogenomic dataset (e.g. CPTAC) to determine correlations between protein levels in primary breast tumours would be valuable here.

Response: We analysed the correlation of USP35 and BRD4 protein levels from the CPTAC dataset. Although USP35 protein level was positively associated with BRD4 protein level ,this

association was not statistically significant (Figure S7A). This is possibly due to the fact that CPTAC dataset contains tumor samples of different subtypes of breast cancer. Importantly, using immunohistochemistry, we detected the a statistically significant positive correlation between USP35 and BRD4 protein levels in ER+ breast tumors (Figure 4G, H).

4. Figure 5 – Title "BRD4 inhibits ferroptosis" is not justified by the data within the figure. Although the BRD4 inhibitor JQ1 increases lipid ROS levels, an additional experiment showing sensitivity of BRD4 shrRNA cells to RSL3-mediated killing would strengthen this (perhaps a colony formation assay).

Response: Thanks for the reviewer raised the questions. We examined the effect of RSL3 treatment in MCF-7 and ZR-75-1 cell lines expressing BRD4 shRNA. RSL3 enhanced lipid ROS level induced by BRD4 knockdown (Figure S8A), and further decreased the cell growth inhibited by BRD4 knockdown (Figure S8B) in both cell lines.

5. The authors state that USP35 is upregulated by estrogen signaling, however it would be good to determine how dependent the USP35-BRD4-SLC7A11 mechanism is on ER status and estrogen sensitivity. Does USP35 over-expression in an ER- cell line (e.g. MDA-MB-231) also render cells resistant to ferroptosis?

Response: The reviewer raised an interesting question. Therefore, we examined the effects of USP35 on MDA-MB-231 cells by overexpressing USP35 in MDA-MB-231 cells. Overexpression of USP35 partially reversed Erastin's inhibition of cell growth and decreased lipid ROS induced by Erastin in MDA-MB-231 cells (Figure S5), indicating that USP35 expression can render ER- cells resistant to Erastin. However, the knockdown of USP35 did not affect the levels of BRD4 protein in TNBC cell lines (MDA-MB-231 and SUM159PT) (Figure S4C). Furthermore, our new data shown in Figure S4D revealed that USP35 did not interact with BRD4 in these two TNBC cell lines, which were different from what we observed in ER+ breast cancer cells. These results indicate that USP35 expression confers resistance to ferroptosis independent of BRD4 in ER- breast cancer cells. These data further support that the effect of the USP35-BRD4-SLC7A11 axis on ferroptosis is dependent on ER status.

Minor points

1. Figure 2D – is the over-expression really just 15-fold higher than the control? It looks more.

Response: We have re-analyzed the intensity of USP35 bands, and the new quantification is shown in Figure 2D.

2. Line 352 "players"? Needs clarification.

Response: We have changed "players" to "genes".

3. Figure 5D – Did the authors test TNBC tumours? Was the correlation still present?

Response: We examined the correlation of SLC7A11 and BRD4 mRNA levels in the basal subtype of breast cancer from TCGA, and found that the levels of SLC7A11 and BRD4 mRNA are positively correlated (Fig. S7B).

4. Figure 7D – The photographs of mice contribute little to the data so should be removed.

Response: The photographs of mice have been removed from Figure7.

1. Li, Y. et al. 7-Dehydrocholesterol dictates ferroptosis sensitivity. Nature. 2024 Feb; 626(7998): 411-418.
2. Cui, W. et al. Gut microbial metabolite facilitates colorectal cancer development via ferroptosis inhibition. Nat Cell Biol. 2024 Jan;26(1):124-137.
3. Qiu, S. et al. Mitochondria-localized cGAS suppresses ferroptosis to promote cancer progression. Cell Res. 2023 Apr;33(4):299-311.

Reviewer #2 (Remarks to the Author):

The manuscript by Cao et al. report that USP35, commonly upregulated in ER+ breast cancers, contributes to tumour growth via the inhibition of ferroptosis. Knockdown of USP35 in ER+ breast cancer cell lines increased several markers of ferroptosis, whilst USP35 overexpression reduced ferroptosis-mediated cell death. The authors uncover a potential mechanism for this effect, with USP35 interacting with and deubiquitinating BRD4, which leads to further upregulation of the cysteine transporter SLC7A11. Interactions were uncovered between USP35-BRD4, USP35-SLC7A11 and BRD4-SLC7A11 in cell lines to support this feedback loop. An ER+ breast cancer in vivo model provided further evidence for the role of USP35 in promoting tumour growth. This is a well conducted article that has used appropriate methodology to investigate an interesting and novel area of research that will provide valuable information for the scientific community.

Response: We thank reviewer 2 for the overall positive assessment of our study and all the constructive suggestions.

Major points to address:
1) In Figure 1C, especially for ZR-75-1, levels of cell viability look very similar following apoptosis or necrosis inhibition despite presentation as statistically significant. It would informative to investigate markers of apoptosis or necrosis following USP35 knockdown to confirm that ferroptosis is the only cell death mechanism inhibited by USP35.

Response: The effect of USP35 knockdown on apoptosis was determined by examining changes in the level of cleaved PARP, a marker of apoptosis. According to data from western blot analysis, knockdown of USP35 did not increase apoptosis of ER+ breast cancer cells (Figure S1A). Serine/threonine kinase RIP1 plays a prominent role in necroptosis, and its activity is controlled by phosphorylation. MLKL serves as the necroptotic executioner to initiate lytic cell death. Knockdown of USP35 did not significantly increase levels of pRIP1 and pMLKL in ER+ breast cancer cells (Figure S1B). These data indicated that knockdown of USP35 exclusively induced

ferroptosis in ER+ breast cancer cells.

2) The authors state that USP35 inhibits ferroptosis independently of BRD4 in TNBCs at line 406. It would be worthwhile to investigate if USP35 and BRD4 interact in a similar matter in TNBC cells as in ER+ breast cancer cells. It would also be interesting to see if TCGA analysis could be conducted on TNBC samples to investigate the association of USP35/ferroptosis and BRD4/SLC7A11 in this cancer type. The authors should discuss any potential evidence/theories for why ferroptosis would be inhibited in different ways by USP35 in these cancer types.

Response: Thanks for the reviewer raised an interesting question. We examined the interaction between USP35 and BRD4 using immunoprecipitation in TNBC cells. The results revealed that USP35 did not interact with BRD4 in MDA-MB-231 and SUM159PT cells (Figure S5D), contrasting with our observations in ER+ breast cancer cells. This evidence further supports that USP35 inhibits ferroptosis independent of BRD4 in TNBC cells.

The association between USP35 and ferroptosis was analyzed in the TNBC cohort from the TCGA database. However, USP35 was not significantly associated with ferroptosis in TNBC (Figure S2), suggesting that USP35 does not play a more general role in regulating ferroptosis in TNBC. But there was a positive correlation between BRD4 mRNA level and SLC7A11 mRNA level in TNBC subtypes (Figure S7B).

We speculate that ER status may play a critical role of USP35 in inhibiting ferroptosis in different ways in the ER+ and ER- breast cancer cells. A published study has showed that BRD4 is important for ERα regulated gene transcription by occupying the estrogen response elements enriched for H3K27ac (PMID: 25017071). Our previous study demonstrated that USP35 via interaction with ERα enhances ERα mediated gene transcription in ER+ breast cancer cells (PMID: 34131114). Since USP35 also interacted with BRD4 (Fig. 4D, E), it is possible that BRD4 together with USP35 and/or ERα regulate the transcription of SLC7A11 in ER+ breast cancer cells. We discussed this point in the second to the last paragraph of the discussion section in the original version of our manuscript.

Our data also showed that USP35 inhibits ferroptosis independent of BRD4 in TNBC cells. USP35 may target one of the many other key proteins regulating ferroptosis in some subtypes of TNBC, which certainly deserve further investigation in the future since the current study is to understand how USP35 regulate ferroptosis in ER+ breast cancer cells. We have added this point to the second to the last paragraph of the discussion section in the revised manuscript.

3) Is there any existing evidence for inhibited ferroptosis in ER+ breast cancer? If so, this should be discussed. Is there any literature indicating why ferroptosis would be selectively targeted in ER+ breast cancers? More detail should be provided on the current usage and how the results from this paper support the use of ferroptosis inducers in the treatment of this disease.

Response: Despite the fact that no ferroptosis inducer has been approved by the FDA for cancer treatment, published papers demonstrated that ER+ breast cancer cell lines are more resistant to

ferroptosis inducers FIN56, Erastin, and RSL3 (PMID: 32937365, 33229547). A recent study has begun to provide some mechanistic insight, showing that estrogen receptor (ER) transcriptionally upregulated the expression of the phospholipid-modifying enzyme MBOAT1, a suppressor of ferroptosis, in ER+ breast cancer cells. Ferroptosis induction combined with ER antagonist significantly inhibited the growth of ER+ breast tumors, even when tumors were resistant to single-agent endocrine therapy (PMID: 37267948). These studies together with our finding support the notion of targeting ferroptosis as a promising option for the treatment of ER+ breast cancer. We have added this paragraph to the first paragraph of the discussion section in the revised manuscript.

Minor points to address:

• Authors should include n numbers for all experiments.

Response: We have included n numbers for all experiments.

• Figure 1I - Pearson's correlation coefficient or equivalent and p value should be included more clearly.

Response: We have included the Pearson's correlation coefficient and p value clearly in Figure 1I.

• MFI values should be included in FACS histogram plots – can be difficult to visualise small differences between peaks of different conditions.

Response: We presented the quantification and statistics on the percentage of positive cells according to many published papers (PMID: 38297130, 38168770, 36864172). In addition, we have expanded the abscissa of the flow cytometry figure, which has enabled the shifts to be seen more clearly. Furthermore, we presented all the MFI values for fluorescence in Figure S9.

• Figure 2A – scale bars should be included for cell viability – are they the same as Figure 2E? IF so, this should be consistent throughout the manuscript.

Response: Scale bars in Figure 2A was different from in Figure 2E. The CCK8 assay was used in Figure 2A, and colony formation assay was used in Figure 2E. We have changed the scale bars to Absorbance (OD450nm) for CCK8 assay, and Absorbance (OD540nm) for colony formation assay.

• Line 320: remove "to" in "to new protein synthesis"

Response: We have removed "to" in Line 357, 358.

• Line 384; Remove "our" between "previous findings"

Response: We have removed "our" between "previous findings" in Line 427.

• Figure 7G – labelled IHC plots incorrectly, 2x USP35+vehicle conditions.

Response: We have corrected this labeling mistake in Figure 7G.

• Could not find a reference to Figure 8 in the text.

Response: We have referenced Figure 8 in the last paragraph of the discussion section in the revised manuscript, in Line 511.

Reviewer #3 (Remarks to the Author):

This manuscript by Cao et al. investigates the role of USP35 in estrogen receptor-positive (ER+) breast cancer, with a focus on ferroptosis, a form of cell death that has garnered significant attention in the research community. While anti-estrogen therapies have improved survival rates, around 30% of patients do not initially respond, highlighting the importance of exploring new treatments for resistant breast cancers. The authors demonstrate that USP35 inhibits ferroptosis by interacting with and stabilizing BRD4, a protein that regulates SLC7A11, which in turn prevents ferroptosis and promotes cancer cell growth. The findings suggest that targeting USP35, in combination with a ferroptosis inducer, may offer a promising therapeutic strategy for ER+ breast cancer resistant to endocrine therapies.

The authors have conducted a thorough literature review, referencing recent publications that support their findings in other cancer types. However, they missed citing Yan et al. 2023 in Nature Communications, which establishes a link between SLC7A11 and ferroptosis.

Response: We thank reviewer 3 for all the constructive suggestions. We have added the Yan et al reference (doi: 10.1002/mco2.337) in line 493 in the revised manuscript.

For the manuscript to be more convincing, the following concerns regarding rigor and reproducibility should be addressed:

Specific Comments on Rigor and Reproducibility:

1.Data Presentation: All bar graphs should include individual data points to allow for better assessment of sample size and distribution (currently, only Figure 7 displays individual points).

Response: We have included individual data points for all of the bar graphs.

2.Statistical Analysis: Unpaired t-tests should not be used unless data follow a normal distribution. Given the potentially low sample sizes, the Mann-Whitney test may be more appropriate.

Response: According analysis, our data approximates a normal distribution. In addition, our

experiments were repeated independently 3 times, and similar result was obtained with statistical difference each time. Therefore, we believe that such analyses for comparing data between two group using unpaired t-tests provided enough robustness to our findings.

3.Materials and Methods: The section lacks sufficient detail for reproducibility. Specific issues include:

Culture Conditions: Missing information on culture media, drug vehicles, cell seeding numbers, and experimental timelines.
References: Avoid vague statements like "previously described," which require readers to consult older references.
Reagent Details: The dilution used for BRD4 is not specified.
Immunoprecipitation (IP): Details such as protein quantities, method specifics, and IgG control references are absent.
Flow Cytometry (FACS): Unclear whether cells are only resuspended in PBS—no mention of FACS buffer or live/dead dyes to exclude non-viable cells.

Response:
Culture Conditions: We have added the information on culture media, drug vehicles, cell seeding numbers, and experimental timelines.

References: We have added detailed information in the sections of "Cell lines and reagents", "Retrovirus/lentivirus production and viral infection", and "Coimmunoprecipitation and immunoblotting".

Reagent Details: The dilution used for BRD4 was 1:1000, and it was added in line 194, 195.
Immunoprecipitation (IP): The detailed information of IP was added in the section of "Coimmunoprecipitation and immunoblotting".
Flow Cytometry (FACS): The cells are only resuspended in PBS, and we exclude non-viable cell through setting the gate. We added the information of gating strategy in Figure S10.

4.IHC Scoring: A supplemental figure should be provided, showing examples of each scoring category (0/1/2/3) to help readers appreciate the differences.

Response: We have provided the representative figure of each scoring category (0/1/2/3) in Figure S9.

5.TCGA Data: The datasets used for TCGA analysis should be clearly cited.

Response: We have cited the datasets used for TCGA analysis in line 249.

6.Experimental Replicates: Biological and technical replicates (n values) should be specified in the figure legends.

Response: We have specified the biological and technical replicates (n values) in the figure legends.

7.Correlation Data:

Figure 1, panel I, lacks the correlation coefficient, which is essential to assess the described negative correlation.
Figure 4, panel H, shows a discrepancy between the indicated sample size (n=18) and the number of dots present (only 11).
Figure 4, panel D, also lacks a correlation coefficient.

Response:
We have added the correlation coefficient of Figure 1I.
In Figure 4H, the sample size exactly was 18, some points were overlapped, making it looks like only 11 dots present.
Regarding Figure 4D, we do not understand the question since it is a simple IP/western blot figure.

By addressing these issues, the manuscript will significantly improve in terms of scientific rigor and reproducibility, enhancing its overall impact.

Reviewer #3 (Remarks to the Author):

I would like to sincerely thank the authors for their thoughtful and thorough responses to my comments and concerns. Their detailed explanations and revisions have significantly enhanced the clarity and rigor of the manuscript. These improvements have strengthened the manuscript's impact and contributed meaningfully to the field.
However, I am still not convinced of the use of the T-test for analyzing the small sample size. Three points per condition seems relatively small to assess the normality of the data. This is my only remaining concern about this study.

Response: We appreciate the reviewer for raising this point, which regards Fig 2D, 3B, 5A, 5C, and 5I. For Figure 5A, we used the Mann-Whitney test to analyze the data in the revised manuscript. For Figure 5C, the data points had a normal distribution confirmed by the Shapiro test, so we used the T-test. Figure 2D, 3B, and 5I are quantified data of western blot bands. In each of these figures, the three data points for each condition were from three independent experiments, not 3 measurements in one single experiment. In addition, the vector control was set as 1, so the three data points do not fit to a normal distribution, which is not suitable to be analyzed by the Mann-Whitney test that requires sample size larger than three. This is the reason why we used the T-test for figure 2D, 3B, and 5I. We also noted that ***Communications Biology*** *recently published manuscripts using T-test for analyzing data with three data point, such as PMID: 39558086; PMID: 39627458; PMID: 39613949; 39638898). We hope that these explanations help alleviate reviewer's concerns.*