nature portfolio

Peer Review File

TET1 as a master regulator controlling GPX4-dependent and-independent ferroptosis surveillance in acute myeloid leukemia

Corresponding Author: Dr Xi Jiang

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.
Parts of this Peer Review File have been redacted as indicated to maintain the confidentiality of unpublished data.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The paper by Yang et al. introduces a novel regulator of ferroptosis sensitivity in human cells. The authors demonstrate that TET1, a protein responsible for converting the epigenetic marker m5C to mh5C, plays a key role in modulating two distinct pathways involved in ferroptosis resistance: the GCLC-GSH-GPX4 and NFkB-GCH1 pathways. Overall, the paper is scientifically robust, presenting ample data to support its claims. The newly identified pathway is highly relevant for ferroptosis research and could provide valuable insights for the development of future ferroptosis-targeted therapies. While the manuscript is solid, it would greatly benefit from some language editing to improve clarity in several sections. Finally, the authors should address a few concerns raised in the review.

Major concerns:
• Since the MTT assay relies on the metabolic activity of cells, please provide a secondary readout of viability, such as AnnexinV negative staining.

• Ensure that the y-axis goes to 0 (Fig. 4J, 4K) and that it is continuous and not separated (Fig. 3H, 3I).

• Can TET1 regulate GCH1 directly? Can the authors perform ChIP-qPCR data to address this question (similar to Fig2f and Fig 5c)?

• The use of a t-test for comparing datasets with multiple variables (e.g., Fig2 n-o, Fig 5k-m) may not be the most appropriate statistical approach. Could the authors clarify their rationale for this choice and explain why a two-way ANOVA (+ some post-hoc) was not used instead?

• It is unclear whether a true correlation exists in Fig 1C. The authors should show the data as a correlation plot (with the appropriate statistics) to better match the text in lines 134-136.

• Please provide GCLC mRNA levels in THP1-GCLC-OE (related to Fig 2J).

• The use of different models is appreciated, but it's often confusing why the authors switch between models. For example, why is Fig 2h and i in different cell lines?

• The authors overuse the term "obvious synergistic effects" in figures that often show non-synergistic effect (e.g., Fig2L, Fig3e-g)

• Please clarify if Fig.3 h-i data was performed in vivo (BSO/plpC administered to live mice) or if the treatment was added to isolated cells in cell culture plates. If the latter is correct, the use of the term "ex vivo" would be more appropriate.

• Fig4: Does shGPX4 abrogate the effects of TET1 compared to shCtrl? Does TET1-OE or KD affects enzymatic GPX4 activity?

• Fig 5p: including the role of GCH1-BH4 in ubiquinol and alpha-tocopherol regeneration in the schematic seems appropriate since the paper does not provide direct evidence that GCH1's effect occurs directly via BH4 on lipid peroxides.

• Tet1 activity (conversion of 5mC to 5hmC) is a Fe+2-dependent oxidation reaction. Can the redox state of the cell affect TET1 activity?

Minor concerns:

• It would be beneficial to briefly discuss in the Introduction the functional differences between m5C and mh5C. How do these epigenetic markers differentially influence gene expression?

• Please Lines 1-2: The sentence "Ferroptosis […] is closely associated with cancer" is not very informative. Please improve this section by clarifying how they are connected, whether they promote/inhibit carcinogenesis and its possible connections to therapy.

• Please provide some background information about MLL-AF9 and AML-ETO9a as this may not be common knowledge for most readers

• When cell line names are displayed in figures, additional labeling reminding the readers of which cells are resistant/sensitive would be useful. That comment applies especially to Fig 2k, but would be useful all across the paper.

• Also in Fig3 h-i, please clarify what the stars (statistical significance) symbols are comparing.

• Line 198 says that GSH/GSSG ratio was assessed, but Fig 3a-l show "GSH levels", not the ratio. Please clarify if this is the ratio, reduced GSH, total glutathione (GSH+GSSG), or something else.

• Line 214: to improve clarity to readers, it might be worth elaborating on the reason for performing tracing metabolomics with 13C5,15N2-Gln instead of a regular unlabeled metabolomics. Please also clarify whether the metabolites detected (GSH and y-Glu-di/tripeptides) are heavy-labeled.

• Fig 5m: please provide more information about this protocol. Some abbreviations, like MSCV-PIG, are not described or found anywhere else in the manuscript.

• It is unclear to me why the authors label Fig 6i-L (and their legends) with the term "PDX" if only established cell lines were used for the xenograft models.

Textual corrections:

• Line 80: FSP-1 is described as dihydroorotate dehydrogenase
• Figure 3k: The star symbol seems to be misplaced below the line
• Supplementary Fig 1c-d: It looks like some of the bars go beyond the maximum value of the Y axis. Please adjust so the real value can be displayed
• Line 286: I believe the authors mean TET1, and not TET2

(Remarks on code availability)
n/a

Reviewer #2

(Remarks to the Author)
In this manuscript, the authors investigated TET1-mediated ferroptosis resistance in cancer. They identified TET1 as a key epigenetic modulator that promotes DNA 5hmC at the GCLC locus, enhancing both canonical glutathione synthesis and non-canonical γ-glutamyl-peptide accumulation. Furthermore, they revealed a GPX4-independent resistance mechanism via TET1/NFκB-mediated upregulation of GCH1, thereby expanding the known ferroptosis defense pathways. The authors also demonstrated the synergistic efficacy of low-dose ferroptosis inducers (e.g., erastin) with TET1/GCLC/GCH1 inhibitors in both ferroptosis-sensitive and -resistant cancers. While therapeutic synergy is suggested, data from immunocompetent models are needed to assess interactions within the immune microenvironment and to determine whether and how in vivo treatments prolong mouse survival by inducing ferroptosis of cancer cells. Additionally, the study lacks primary patient sample analysis to correlate TET1/5hmC levels with ferroptosis resistance, and it is unclear if TET1 inhibition affects other 5hmC-dependent processes beyond GCLC/GCH1. The limited comparison to other epigenetic regulators (e.g., DNMTs) further weakens the claims of TET1 specificity.

Overall, this work advances ferroptosis research by uncovering an epigenetic layer of regulation. While translational research requires further in vivo validation of target specificity, the combination strategy aligns with emerging trends in targeting parallel resistance pathways.

There are some major concerns:

Figure 1a. TET1 was identified through RNA-seq using the Kasumi-1 cell line compared to the MV4;11 cell line. However, different cell lines, particularly THP1, were used in various downstream experiments. Including Kasumi-1 and MV4;11 as positive and negative controls, respectively, throughout the study would be beneficial.

Figure 1c:
1. How were basal expression levels of TET1 measured in 30 samples? qPCR (?): How many biological replicates? P-value? How about the TET1 protein levels?
2. TET1 showed extremely low expression in MV4;11, suggesting that MV4;11 might be independent of TET1. However, in Supplementary Figure 1J, TET1 knockdown by shRNAs (~50% knockdown efficiency) significantly reduced the cell viability of MV4;11. There is an inconsistent observation between TET1 expression and function.
3. It is unclear how AML subtypes and driver mutations are related to TET1-mediated ferroptosis resistance.
4. Low TET1 was observed in MLL-AF9/MLL-AF4 cell line. MOLM13 (MLL-AF9) and MV4;11 (MLL-AF4) exhibited minimal TET1 expression and showed high sensitivity to the GPX4 inhibitor RSL3 (ferroptosis inducer). THP1 (MLL-AF9) has low TET1 but is RSL3-resistant. These observations are contrast with primary AML data, where genome-wide studies consistently report TET1 upregulation in primary MLL-rearranged AML, and TET1 cooperates with MLL fusions to drive leukemogenesis.

The key inconsistencies of TET1 expression between human cell lines and primary MLL AML implicate cell line limitations. Established AML cell lines (e.g., MOLM13, THP1) often lose epigenetic heterogeneity seen in primary samples, potentially explaining aberrant TET1 downregulation despite MLL rearrangements. Ferroptosis sensitivity in these lines may reflect compensatory pathways (e.g., low GSH synthesis) unrelated to TET1 status. Primary MLL-rearranged AML relies on TET1-mediated epigenetic regulation (e.g., 5hmC modification at oncogenic loci). Cell lines may bypass this dependency through acquired mutations or culture adaptations. The questions are: do primary MLL-rearranged AML cells with high TET1 exhibit ferroptosis resistance due to the role of TET1 in glutathione metabolism (via GCLC)? Conversely, does TET1-low status (e.g., TET1 knockdown) correlate with RSL3 sensitivity in primary AML patient-derived xenografts?

Figure 1f,g: These figures illustrate the in vitro treatment of mouse AML cells, derived from Tet-/- mice, with RSL3. It is important to clearly state this in the manuscript text to avoid any confusion with in vivo treatment. Additionally, would the authors consider conducting in vivo treatment of AML Tet-/- mice with RSL3?

Figure 2d,e: Would knockdown and overexpression of TET1 change GCLC protein levels?

• Iron levels are a crucial determinant of ferroptosis. What are the iron levels in human and mouse cells treated with RSL3?
• How were the RNA-seq data analyzed, and what cutoff criteria were used
• If TET1 controls cancer cell susceptibility to ferroptosis in both GPX4-dependent and -independent mechanisms, which pathway is predominant and how do these two pathways interact? If one pathway is inhibited, would the other compensate?
• Most experiments have been conducted using human cell lines, which do not fully recapitulate patient conditions. It is crucial to determine whether and how TET1-mediated ferroptosis resistance applies to patient-derived xenograft (PDX) models from primary AML samples and how TET1-mediated ferroptosis resistance is linked to AML subtypes and their driver mutations.

Minor concerns:
• Please state the number of biological replicates performed across all experiments, as well as the method used to calculate the p-value. Some statistical analyses appear to be incorrect. For example, Figures 1f, 1g, 1j, and 1k might require two-way ANOVA, while Figures 1e, 1h, and 1i might require one-way ANOVA, and so on.
• Figure 1j is difficult to read and needs to be presented more clearly. Additionally, it lacks statistical analysis.

(Remarks on code availability)

Reviewer #3

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

(Remarks on code availability)

Reviewer #4

(Remarks to the Author)
This manuscript by Yang et al. presents a compelling and well-executed study identifying TET1 as a central regulator of ferroptosis resistance in cancer, with a particular focus on acute myeloid leukemia (AML). The authors demonstrate that TET1 modulates both GPX4-dependent and -independent ferroptosis surveillance pathways by transcriptionally regulating GCLC and GCH1 via 5hmC-mediated epigenetic remodeling and NF-κB signaling. The use of shRNA knockdowns, overexpression constructs, ChIP-seq, 5hmC-seq, RNA-seq, in vivo AML mouse models, and human AML samples provides robust experimental support. This study brings novel insights into the epigenetic control of ferroptosis and highlights TET1 as a potential therapeutic target in ferroptosis-resistant AML. However, several critical points require clarification and additional data to ensure the broader validity and mechanistic precision of the conclusions:

Major Point:

1. The manuscript title refers to TET1 as a “master regulator of ferroptosis surveillance in cancer.” However, the study is almost exclusively focused on acute myeloid leukemia (AML), as evidenced by the use of human AML cell lines, murine AML models (MA9 and AE9a), and AML patient samples. TET1 has been shown to play divergent roles in hematological malignancies—tumor-suppressive in B-cell acute lymphoblastic leukemia (B-ALL) and oncogenic in T-cell ALL (T-ALL). Thus, the generalization to "cancer" may overstate the findings. A more accurate title, such as “TET1 as a master regulator of ferroptosis surveillance in AML”, would better reflect the specificity and experimental scope of the study.

2. AML is sustained by a subset of self-renewing leukemic initiating cells (LICs), which are thought to mediate therapy resistance and disease relapse. While the study thoroughly characterizes TET1’s role in bulk AML populations, it remains unclear whether TET1 regulates ferroptotic susceptibility in LICs. Investigating this would be of high translational relevance. Specifically, evaluating LIC frequency and function after co-treatment with RSL3 and TET1 inhibitors (e.g., Bobcat339, BSO, DAHP) in LIC-enriched compartments or serial transplantation assays could offer valuable insights.

3. According to the methods, the authors overexpressed the catalytic domain (CD) of TET1, which lacks the N-terminal CXXC zinc finger DNA-binding domain essential for site-specific chromatin targeting. While the catalytic domain can mediate hydroxymethylation, it may not fully recapitulate the genomic localization or gene regulatory functions of endogenous full-length TET1. Therefore, conclusions regarding direct transcriptional activation of target genes (e.g., GCLC) would be more robust if validated by overexpressing full-length TET1 and performing ChIP-qPCR or luciferase reporter assays to confirm target engagement.

4. The manuscript establishes a link between TET1 and ferroptosis-related genes (e.g., GCLC, GCH1) in cell lines. To enhance translational relevance, the authors should extend this analysis to primary patient datasets, such as TCGA AML and solid tumor cohorts, using tools like cBioPortal. Assessing whether TET1 expression correlates with GCLC and GCH1 expression in patient-derived transcriptomic data would validate these findings in clinically relevant settings.

5. TET2 and TET3 share overlapping enzymatic functions with TET1 in catalyzing 5mC to 5hmC conversion and are often co-expressed in hematopoietic cells. It is plausible that knockdown or overexpression of TET1 could affect the expression or activity of TET2/TET3, which might confound interpretation of TET1-specific effects. The authors should therefore evaluate TET2 and TET3 expression in TET1 knockdown and overexpression models to exclude functional redundancy or compensatory regulation.

6. The manuscript reports correlations between TET1 expression levels and ferroptosis sensitivity in various cancer cell lines. However, the methodology for quantifying TET1 expression is not clearly stated. The authors should clarify whether RNA-seq or qRT-PCR was used. If RNA-seq was employed, it is important to report the normalization metric (e.g., TPM, FPKM), source of the dataset (e.g., in-house sequencing, CCLE, TCGA), and the statistical method (e.g., Pearson/Spearman correlation) used in the analysis.

7. To increase confidence in the RNA-seq-derived expression data, the authors should validate TET1, GCLC, and GCH1 expression levels in a representative panel of ferroptosis-sensitive and -resistant cell lines using qRT-PCR. This step is standard in transcriptomic studies and serves to confirm the accuracy and reproducibility of computational findings.

8. Transcript abundance does not always correlate with protein levels due to post-transcriptional regulation. The authors should therefore confirm TET1 protein expression using Western blotting across key cell lines used in the study. This would reinforce the mechanistic link between TET1 levels and ferroptosis resistance and clarify any post-transcriptional modulation of TET1.

Minor Points:

9. In lines 286–287, the authors state that “ChIP-q-PCR analysis verified the association between TET2 and the promoter region of NFKB2 (Fig. 5c),” which appears to be a typographical error. Based on the context of the study and the figure legend, the ChIP assay was performed for TET1, not TET2. The authors should correct this to maintain consistency and avoid confusion.

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Reviewer #5

(Remarks to the Author)
In the present study, the authors demonstrate that TET1 enhances resistance to ferroptosis in cancer cells by improving antioxidant defense mechanisms. The knockdown of TET1 increases sensitivity to ferroptosis inducers, such as RSL3, in both ferroptosis-sensitive and -resistant cancer cells, which leads to elevated levels of reactive oxygen species (ROS) and

lipid peroxidation. These effects are reversible with the application of ferroptosis inhibitors. Mechanistically, TET1 transcriptionally activates GCLC, a critical enzyme in glutathione (GSH) synthesis, through 5hmC modifications and promoter binding, thus increasing GSH production and alleviating ferroptotic stress. Additionally, TET1 promotes the synthesis of non-canonical γ-glutamyl-peptides under conditions of cystine deprivation, which supports glutamate scavenging. Importantly, the protective role of TET1 extends beyond dependence on GPX4, indicating its involvement in alternative ferroptosis defense mechanisms, such as the GCH1–BH4 pathway. TET1 regulates the expression of GCH1 via the activation of NFκB signaling, particularly through NFKB2, thereby adding another dimension to its role in resistance to ferroptosis. The TET1/GCLC and TET1/NFκB/GCH1 pathways collectively establish TET1 as a potential regulator of cancer cell susceptibility to ferroptosis. The manuscript is overall interesting and

Suggestions
- Testing GCLC expression and GSH/GSSG levels in NFE2L2 knockout/TET1-high cancer cells could help demonstrate the impact of TET1 in regulating GCLC.
- FSP1 is recognized as one of the major contributors to ferroptosis defense mechanisms, functioning independently of glutathione. In this context, do the TET1-high cancer cell lines shown in Figure 1c rely on TET1 activity to compensate for the lower expression of FSP1? It would be helpful to present the trends in protein expression of FSP1, TET1, and GCH1 across the cancer cell lines used in the study (primarily AML).
- Regarding the investigation of the TET1/GCLC axis in GSH and γ-glutamyl-peptide synthesis (Figure 3), the readout should include not only glutathione levels but also cell viability, given the effects of glutathione depletion. I suggest extending the cystine starvation period beyond 12 hours in TET1 knockout and overexpression cells and assessing whether TET1-OE cells show improved survival due to higher γ-glutamyl-peptide levels.
- To demonstrate that the upregulation of GCH1 activity induced by TET1 and NF-κB protects cells from lipid peroxidation, measuring tetrahydrobiopterin levels while modulating TET1 and NF-κB expression would be beneficial.

Specific points
Line 74-76: The genetic regulation of GSH and γ-glutamyl-peptides biosynthesis has been investigated. I would suggest to mention that NRF2 transcribes GCLC, GCLM, GSR1, xCT (PMID: 28899199) and also GCLC regulates γ-glutamyl-peptides biosynthesis (PMID: 33357455).

Line 80: dihydroorotate dehydrogenase (FSP1) ferroptosis suppressor protein 1 (FSP1)

Line 118-119: To emphasize the correlation between TET1 expression and sensitivity to GPX4 inhibition, it would be better to explain that both RSL3 and ML210 inhibit the enzymatic activity of GPX4, thereby inducing ferroptosis.

Line 696-698: 200 mM cystine supplementation in the cystine/cysteine/methionine/glutamine-free RPMI-1640 seems to be 1000 times higher concentration than in a standard culture medium condition. Is it the correct concentration? Additionally, please confirm the methionine concentration in the medium used for the experiment (100 mM).

Western blots are missing molecular weight mark

Comments on Figures
Figure 1
- Has the knockdown and knockout of TET1 in the cells been confirmed through protein or gene expression analysis? It would be beneficial to present this alongside the phenotypic analysis.
Figure 3
- q) System X System xc-.
- q) The arrow linking glutamate and ferroptosis could result in a misinterpretation that glutamate accumulation is the direct cause of ferroptosis.
Figure 5
p) System X → System xc-.
p) A line is missing to connect glycine to the glutathione synthesis pathway (as shown in Figure 3-q).

Minor points
- Including “TET1-HxD: TET1 catalytically dead mutant” in the Abbreviation section is better.
- This study's main finding is that TET1 contributes to the defense against ferroptosis by regulating the expression of GCLC and GCH1. Incorporating these key points could make the title of the manuscript more specific.

(Remarks on code availability)

Reviewer #6

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

(Remarks on code availability)

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
All concerns have been addressed.

(Remarks on code availability)

Reviewer #2

(Remarks to the Author)
The reviewer acknowledges the authors' efforts in addressing most of the questions raised. Nevertheless, two questions remain unresolved and require further clarification:

1. The legend of Suppl Fig. 1i states: "Expression levels of TET1 were detected with Western blot (WB) and data were 31 normalized to basal TET1 level of Kasumi-1 cells. (red: ferroptosis-sensitive, blue: ferroptosis-insensitive)."
- Please specify how TET1 protein levels were determined. For better interpretability, please quantify the western blot bands and displaying the relative expression values above each TET1 band in the figure.
- Based on the current WB data, TET1 protein expression does not appear to consistently correlate with ferroptosis sensitivity. For example, Kasumi-1 (blue; ferroptosis-insensitive) shows relatively high TET1 expression compared to MV4;11 (red; ferroptosis-sensitive). Conversely, other ferroptosis-insensitive cell lines such as MIA PaCa-2, ASPC-1, HepG2, and NB4 exhibit low TET1 levels, similar to or even lower than those in MV4;11. Likewise, ferroptosis-sensitive cell lines like H292 and THP1 display high TET1 expression comparable to that of Kasumi-1.

2. For in vivo treatment of PDX models in Fig. 6h-l:
- While CD33 is commonly used to identify AML blasts, it is also expressed on normal human myeloid cells. This overlap can lead to false positives when CD33 is used as a sole marker. To improve specificity, additional AML-associated surface markers should be considered, such as hCD45/mCD45, which were used in the CDX models shown in Suppl Fig. 6n and 6o.
- Could you provide data comparing spleen sizes between treated and control mice, as well as the extent of AML blast cell infiltration into spleen tissue? This would help assess treatment efficacy and disease burden.
- Additionally, could the PDX models be used to investigate whether primary AML cells with high TET1 expression exhibit ferroptosis resistance, potentially due to TET1's role in regulating glutathione metabolism via GCLC?

(Remarks on code availability)

Reviewer #3

(Remarks to the Author)
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(Remarks on code availability)

Reviewer #5

(Remarks to the Author)
Thank you for the opportunity to review the revised manuscript. I am very impressed by the authors' detailed responses and the significant effort they put into conducting additional experiments. I have reviewed the authors' responses and the revised manuscript, and my previous comments have been addressed as follows:

Comment-1: The restoration of GCLC expression and GSH levels by overexpressing TET1 in NFE2L2-KO cells suggests that GCLC might be transcribed by other transcription factors, and this process could be supported by TET1-mediated DNA demethylation of the GCLC promoter region. While the experiment you performed meets my initial suggestion, this new data raises an interesting follow-up question: is TET1's demethylation activity essential for GCLC transcription? This could be investigated by analyzing GCLC expression levels in TET1 knockdown cells treated with an NRF2 activator (e.g., KI696). However, this is a new area of inquiry that is not critical for the current manuscript and does not require new experiments.

Comment-2: The effort on expression analysis with patient-derived cancer samples clarified the positive correlation between TET1 and GCLC. I also appreciate that the authors clarified there is no strong correlation between TET1 and FSP1.
Comment-3: The new experiments in Supplementary Figures 3f and 3g directly address my previous question. The data clearly demonstrates that cell viability under cystine starvation depends on TET1 expression.
Comment-4: All the new experiments have excellently addressed the previous suggestions and have significantly improved the overall quality of the manuscript.
Specific and minor points: Upon reviewing the revised version of the manuscript, I find that the text, figures, schemes, and title are now clearly presented.
Overall, the authors have expanded on their findings with newly conducted experiments. Based on these revisions, I recommend acceptance for publication without further changes.

(Remarks on code availability)
n.a

Reviewer #6

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(Remarks on code availability)

Reviewer #7

(Remarks to the Author)
The manuscript by authors Lingling Yang et al. discovered TET1 governs dual-pathway ferroptosis resistance in AML via both GPX4-dependent and independent axes as a master regulator of ferroptosis susceptibility. Importantly, authors combined TET1 inhibitor and ferroptotic inducer against AML, especially ferroptotic resistant AML, which exhibit potential therapeutic efficacy either in vitro or in vivo. The authors provide more data in this revised manuscript to improve their scientific claims with additional evidence, typically in response to all the peer reviewers' or editor's requests. This strengthens the paper by making the findings more robust, transparent, and reproducible. Therefore, I suggest the current version of the manuscript is acceptable for publication in "Nature Communications".

(Remarks on code availability)

Version 2:

Reviewer comments:

Reviewer #2

(Remarks to the Author)
The authors have addressed my comments and reinforced their findings with additional experiments. I recommend acceptance without further revisions.

(Remarks on code availability)

Manuscript ID: NCOMMS-25-18361-T

Manuscript Title:

TET1 as a master regulator controlling both GPX4-dependent and -independent ferroptosis surveillance in AML

SUMMARY: We sincerely thank the reviewers for their insightful comments and constructive suggestions on our manuscript. In response to their feedback, we have conducted additional experiments and performed further data analysis, leading to substantial revision throughout the manuscript. Notably, we have incorporated multiple patient-derived xenograft (PDX) models to strengthen the study. Utilizing these PDX models (*in vivo*) and primary AML cells collected from patients (*in vitro*), we have not only comprehensively evaluated the therapeutic potential of targeting TET1-mediated ferroptosis surveillance in AML treatment, but also deepened our mechanistic investigation. All modifications and additions to the manuscript are highlighted in **RED TEXT**. Removed content has been deleted entirely from the manuscript. Our point-by-point responses to the reviewers' comments are provided below. Each original comment is presented, followed by our "**RESPONSE**". We believe that the revised manuscript now addresses all reviewers' concerns and hope that it meets the standards for publication in *Nature Communications*.

The following are our point-by-point responses to the Reviewers' comments:

1. Responses to the comments of Reviewer 1:

General Comments from Reviewer 1**:** *The paper by Yang et al. introduces a novel regulator of ferroptosis sensitivity in human cells. The authors demonstrate that TET1, a protein responsible for converting the epigenetic marker m5C to mh5C, plays a key role in modulating two distinct pathways involved in ferroptosis resistance: the GCLC-GSH-GPX4 and NFkB-GCH1 pathways. Overall, the paper is scientifically robust, presenting ample data to support its claims. The newly identified pathway is highly relevant for ferroptosis research and could provide valuable insights for the development of future ferroptosis-targeted therapies. While the manuscript is solid, it would greatly benefit from some language editing to improve clarity in several sections. Finally, the authors should address a few concerns raised in the review.*

RESPONSE: We appreciate Reviewer's careful going over, pertinent comments and positive appraisals. In order to address Reviewer's concerns, we performed additional

experiments and analysis. And the manuscript has been further edited by native speakers. Please see details below.

Comment 1 from Reviewer 1: *Since the MTT assay relies on the metabolic activity of cells, please provide a secondary readout of viability, such as AnnexinV negative staining.*

RESPONSE: We thank Reviewer for the suggestion! Following Reviewer's suggestion, here we performed Annexin V/propidium iodide (PI) staining to provide a secondary readout of cell viability, besides MTT assays. Results show interfering *TET1/Tet1* expression significantly affects RSL3-induced apoptosis, in consistence with MTT readouts, and have been included as Supplementary Figs. 1o, s-w. The apoptosis induction effects of ferroptosis inducer(s) are consistent with previous reports (*Hu et al., Front Cell Dev Biol, 2022; Liu et al., Cell Death & Disease, 2024*).

Comment 2 from Reviewer 1: *Ensure that the y-axis goes to 0 (Fig. 4J, 4K) and that it is continuous and not separated (Fig. 3H, 3I).*

RESPONSE: We appreciate Reviewer's careful going over. The start points of the y-axis of Figs. 4j and k have been adjusted to 0. The break bars of Figs. 3g and h (previously Figs. 3h and i) have been removed.

Comment 3 from Reviewer 1: *Can TET1 regulate GCH1 directly? Can the authors perform ChIP-qPCR data to address this question (similar to Fig2f and Fig 5c)?*

RESPONSE: We thank Reviewer for the very good suggestion! According to the suggestion, we conducted ChIP-Q-PCR analysis, but found no obvious binding between TET1 protein and multiple sites of the *GCH1* gene locus, including the promoter region, 5'UTR, CDS, and 3'UTR. These results indicate that TET1 may not directly regulate the transcriptional activity of *GCH1*. The above negative results have been included as Supplementary Fig. 5b.

Comment 4 from Reviewer 1: *The use of a t-test for comparing datasets with multiple variables (e.g., Fig2 n-o, Fig 5k-m) may not be the most appropriate statistical approach. Could the authors clarify their rationale for this choice and explain why a two-way ANOVA (+ some post-hoc) was not used instead?*

RESPONSE: We thank Reviewer for pointing out the inappropriate statistical approaches. Two-way ANOVA should be used for showing the interaction between two independent variables, while t-test is used to compare one independent variable between exactly two groups. The statistical methods of Figs. 1d, 1f-g, 1j-k, 2h-j, 2l-o, 5j-q, 6f-g and Supplementary Figs. 1l-n (previously Supplementary Figs. 1j-l), 1p,

2h-i, 2l-m (previously Supplementary Figs. 2h-k), 3f-g, 4b-d, 4f-i (previously Supplementary Figs. 4e-h), 4m-n, and 5i have been changed into two-way ANOVA.

Comment 5 from Reviewer 1: *It is unclear whether a true correlation exists in Fig 1C. The authors should show the data as a correlation plot (with the appropriate statistics) to better match the text in lines 134-136.*
RESPONSE: Thanks for the suggestion! To better clarify the correlation extent ($r=0.743$, $P<0.001$), a correlation plot has been included beside the correlation curves for Fig. 1c.

Comment 6 from Reviewer 1: *Please provide GCLC mRNA levels in THP1-GCLC-OE (related to Fig 2J).*
RESPONSE: Thanks for Reviewer's suggestion! *GCLC* mRNA levels determined through Q-RT-PCR have been included beside the previous Fig. 2j.

Comment 7 from Reviewer 1: *The use of different models is appreciated, but it's often confusing why the authors switch between models. For example, why is Fig 2h and i in different cell lines?*
RESPONSE: We appreciate this question. According to Fig. 1c and Supplementary Figs. 1a-d, MV4;11 is relatively sensitive, while Kasumi-1 is relatively resistant to ferroptosis. Therefore, we tested the desensitizing effect of *GCLC* overexpression in MV4;11, and the sensitizing effect of *GCLC* knockdown in Kasumi-1. In order to avoid confusion, results of *GCLC* overexpression in Kasumi-1 and *GCLC* knockdown in MV4;11 have been added as Supplementary Figs. 2h and i. These results further confirm the ferroptosis sensitizing effect of *GCLC* knockdown even in ferroptosis sensitive MV4;11 cells, but *GCLC* overexpression shows no obvious effects in ferroptosis resistant Kasumi-1 cells.

Comment 8 from Reviewer 1: *The authors overuse the term "obvious synergistic effects" in figures that often show non-synergistic effect (e.g., Fig2L, Fig3e-g)*
RESPONSE: We thank Reviewer for the comment. Although statistical significance was found between the combined treatment group and single agent treated group, the robust cell viability suppression exerted by full-dose ferroptosis inducers and BSO partially masked their potential synergistic effects, especially in ferroptosis-sensitive cells, such as MV4;11 cells and mouse *MA9*-AML cells. Therefore, for these cells, we further cut down the dose of RSL3 from 250 nM to 100 nM, BSO from 100 μM to 50 μM, and Erastin from 10 μM to 5 μM, and repeated the combinational treatment experiments. In mouse *MA9*-AML cells (Fig. 2l), BSO alone reduced the viability to

68.1% (DMSO treated control as 100%); RSL3 alone reduced the viability to 68.2%; BSO+RSL3 further reduced cell viability to 42.5%. According to two-way ANOVA analysis, all intergroup differences, i.e., BSO v.s. Ctrl, RSL3 v.s. Ctrl, BSO+RSL3 v.s. BSO, and BSO+RSL3 v.s. RSL3, are statistically significant. In MV4;11 cells (Fig. 3d, previously Fig. 3e), BSO alone reduced the viability to 66.8%; Bobcat339 alone reduced the viability to 82.8%; BSO+ Bobcat339 further reduced cell viability to 14.1%. According to two-tailed t-test analysis, all intergroup differences, i.e., BSO v.s. Ctrl, Bobcat339 v.s. Ctrl, BSO+ Bobcat339 v.s. BSO, and BSO+ Bobcat339 v.s. Bobcat339, are statistically significant. As is shown in Figs. 3e and f (previous Figs. 3f and g), BSO, Bobcat339 or Erastin alone reduced GSH level to 55.1%, 68.0% or 69.2%, respectively, while Erastin+BSO further reduced GSH to 7.34%, Erastin+Bobcat339 to 24.5%, with statistical significance as comparing with single drug treated groups (two-tailed t-test). These results suggest potential strengthening effects of the combinational administration with BSO and ferroptosis inducer, Bobcat339 and ferroptosis inducer, or BSO and Bobcat339, as compared with a single drug alone. To avoid any controversy, we have removed the words "synergistic effects" from description of Figs. 2l, 3d-f in the manuscript.

Comment 9 from Reviewer 1: *Please clarify if Fig.3 h-i data was performed in vivo (BSO/plpC administered to live mice) or if the treatment was added to isolated cells in cell culture plates. If the latter is correct, the use of the term "ex vivo" would be more appropriate.*

RESPONSE: We thank Reviewer for the suggestion. Figs. 3g-h (previously Figs. 3h-i) were performed in mouse AML cells collected from leukemic mice transplanted with Mx1-Cre$^{+/-}$*Tet1*$^{fl/fl}$*-MA9* cells. Cells were then cultured and treated with BSO and/or PIPC *in vitro*. To avoid confusion, the "*ex vivo*" description has been included in the manuscript, and related figure legends have been revised according to Reviewer's suggestion.

Comment 10 from Reviewer 1: *Fig4: Does shGPX4 abrogate the effects of TET1 compared to shCtrl? Does TET1-OE or KD affects enzymatic GPX4 activity?*

RESPONSE: We thank Reviewer for the very good questions! Knockdown of *GPX4* reduced *TET1* overexpression-induced ferroptosis resistance. *TET1* overexpression or knockdown showed no obvious effects on GPX4 enzymatic activity as determined by NADPH levels. The above results have been added into the manuscript as Supplementary Figs. 4d, m, and n.

Comment 11 from Reviewer 1: *Fig 5p: including the role of GCH1-BH4 in ubiquinol and alpha-tocopherol regeneration in the schematic seems appropriate since the paper does not provide direct evidence that GCH1's effect occurs directly via BH4 on lipid peroxides.*

RESPONSE: We thank Reviewer for the good suggestion! We performed additional experiments and found that ectopic expression of either *TET1* or *NFKB2* led to a significant elevation of intracellular BH4 levels and a decrease of RSL3-induced ROS accumulation (see new Fig. 5p and Supplementary Fig. 5f). Conversely, knockdown of *TET1* or *NFKB2* resulted in a marked reduction of BH4 production and an increase of RSL3-induced ROS accumulation (Fig. 5q and Supplementary Fig. 5g). These findings suggest a previously unidentified regulatory axis wherein both TET1 and NFKB2 critically modulate GCH1 expression and/or function, thereby unveiling the existence of the TET1/NFκB signaling/GCH1 axis in cancer ferroptosis regulation.

Comment 12 from Reviewer 1: *Tet1 activity (conversion of 5mC to 5hmC) is a Fe+2-dependent oxidation reaction. Can the redox state of the cell affect TET1 activity?*

RESPONSE: We thank Reviewer for this question. We tested the relationship between the redox state and TET1 activity. In multiple AML cell lines, RSL3 treatment showed no obvious influence on *TET1* expression or global DNA 5hmC level (Supplementary Fig. 1j, k), indicating the correlation between *TET1* expression and ferroptosis susceptibility may not be determined by cellular oxidation state.

Minor concerns from Reviewer 1:

• *It would be beneficial to briefly discuss in the Introduction the functional differences between m5C and mh5C. How do these epigenetic markers differentially influence gene expression?*

RESPONSE: We sincerely appreciate Reviewer's careful going over and very good suggestions. A backgroud introduction about 5mC, 5hmC and TETs induced epigenetic regulation on gene expression has been added into Introduction, lines 96-105.

• *Please Lines 1-2: The sentence "Ferroptosis […] is closely associated with cancer" is not very informative. Please improve this section by clarifying how they are connected, whether they promote/inhibit carcinogenesis and its possible connections to therapy.*

RESPONSE: A brief backgroud introduction about the relationship between ferroptosis and cancer has been added into Introduction, line 63-66.

• *Please provide some background information about MLL-AF9 and AML-ETO9a as this may not be common knowledge for most readers*
RESPONSE: A backgroud introduction has been included on Page 8, lines 174-177.

• *When cell line names are displayed in figures, additional labeling reminding the readers of which cells are resistant/sensitive would be useful. That comment applies especially to Fig 2k, but would be useful all across the paper.*
RESPONSE: Additional labelings of "sensitive/insensitive" have been added into all related figures across the manuscript, including Fig. 2k.

• *Also in Fig3 h-i, please clarify what the stars (statistical significance) symbols are comparing.*
RESPONSE: All star symbols and comparing groups have been clearly labeled now in Figs. 3g-h (previously Figs. 3h-i).

• *Line 198 says that GSH/GSSG ratio was assessed, but Fig 3a-I show "GSH levels", not the ratio. Please clarify if this is the ratio, reduced GSH, total glutathione (GSH+GSSG), or something else.*
RESPONSE: In Figs. 3a-h and 3n (previously Figs. 3a-i and 3o), the kit name we used for assessing GSH levels was called GSH/GSSG ratio detection kit (Abcam), and shown are direct GSH readouts normalized to negative control groups, not GSH/GSSG ratios. In Fig. 3i, GSH levels were detected with LC-HRMS analysis. We have revised figure legends accordingly to avoid confusion.

• *Line 214: to improve clarity to readers, it might be worth elaborating on the reason for performing tracing metabolomics with 13C5,15N2-Gln instead of a regular unlabeled metabolomics. Please also clarify whether the metabolites detected (GSH and y-Glu-di/tripeptides) are heavy-labeled.*
RESPONSE: Both unlabeled and heavy-labeled metabolites detection readouts were included. The unlabeled metabolomics measures static pool sizes, whereas $^{13}C_5$, $^{15}N_2$-Gln isotope tracing allows direct tracking of glutamate-derived carbon and nitrogen incorporation into GSH and γ-Glu-di/tripeptides, distinguishing them from other potential sources, and thus provides dynamic flux information, revealing how glutamine contributes to GSH and γ-Glu-di/tripeptides synthesis under certain conditions. To clarify readouts of the above distinct metabolomics analysis, we further

adjusted Figs. 3i and j (previously Figs. 3j, k), by splitting the labeled and unlabeled readouts. Corresponding descriptions have been added into the manuscript, lines 270-271, 280, 895-900 and figure legends.

• *Fig 5m: please provide more information about this protocol. Some abbreviations, like MSCV-PIG, are not described or found anywhere else in the manuscript.*
RESPONSE: For Fig. 5m, MV4;11 cells were transfected with pLJM1 or *TET1*, and were concurrently treated with DMSO, RSL3 or/and DAHP for 48 hours. Cell viability was detected by MTT. Corresponding figure legends have been added. The full item of MSCV-PIG has been included on Page 12, line 290.

• *It is unclear to me why the authors label Fig 6i-L (and their legends) with the term "PDX" if only established cell lines were used for the xenograft models.*
RESPONSE: Figs. 6i-l have now been replaced with PDX models using patient derived primary AML cells as donor cells. The previous cell line-derived xeno-BMT results are now labeled as "CDX" and moved to Supplementary Figs. 6l-o.

Textual corrections suggested by Reviewer 1:
• *Line 80: FSP-1 is described as dihydroorotate dehydrogenase*
• *Figure 3k: The star symbol seems to be misplaced below the line*
• *Supplementary Fig 1c-d: It looks like some of the bars go beyond the maximum value of the Y axis. Please adjust so the real value can be displayed*
• *Line 286: I believe the authors mean TET1, and not TET2*
RESPONSE: We sincerely appreciate Reviewer's careful going over. All the above typos and mislabeling have been corrected.

2. Responses to the comments of Reviewer 2:
General Comments from Reviewer 2**:** *In this manuscript, the authors investigated TET1-mediated ferroptosis resistance in cancer. They identified TET1 as a key epigenetic modulator that promotes DNA 5hmC at the GCLC locus, enhancing both canonical glutathione synthesis and non-canonical γ-glutamyl-peptide accumulation. Furthermore, they revealed a GPX4-independent resistance mechanism via TET1/NFκB-mediated upregulation of GCH1, thereby expanding the known ferroptosis defense pathways. The authors also demonstrated the synergistic efficacy of low-dose ferroptosis inducers (e.g., erastin) with TET1/GCLC/GCH1 inhibitors in both ferroptosis-sensitive and -resistant cancers. While therapeutic synergy is suggested, data from immunocompetent models are needed to assess interactions within the immune microenvironment and to determine whether and how in vivo*

treatments prolong mouse survival by inducing ferroptosis of cancer cells. Additionally, the study lacks primary patient sample analysis to correlate TET1/5hmC levels with ferroptosis resistance, and it is unclear if TET1 inhibition affects other 5hmC-dependent processes beyond GCLC/GCH1. The limited comparison to other epigenetic regulators (e.g., DNMTs) further weakens the claims of TET1 specificity. Overall, this work advances ferroptosis research by uncovering an epigenetic layer of regulation. While translational research requires further in vivo validation of target specificity, the combination strategy aligns with emerging trends in targeting parallel resistance pathways.

RESPONSE: We thank Reviewer for all the pertinent comments and constructive suggestions. We have included more AML primary samples, employed multiple PDX models using AML patient primary cells as donor cells, and performed additional experiments and analysis to further strengthen both the mechanism and the translational significance of targeting the TET1 axis in ferroptosis induction therapy in AML. Please see point-to-point response below for more details.

"…it is unclear if TET1 inhibition affects other 5hmC-dependent processes beyond GCLC/GCH1"—While our current study focuses on the major established ferroptosis surveillance pathways, we acknowledge that TET1-mediated DNA modifications may potentially regulate additional, yet-to-be-identified ferroptotic defense mechanisms—an intriguing possibility that warrants future investigation. The above clarification has been added into Discussion, Page 19, lines 515-517.

"The limited comparison to other epigenetic regulators (e.g., DNMTs) further weakens the claims of TET1 specificity."— As a matter of fact, roles of other epigenetic modulators, such as DNMTs, remain controversial in cell ferroptosis (*Feng et al., Exp Hematol Oncol, 2024; Yang et al., MedComm, 2023; Li et al., Cell Death Discov, 2021*). Here we compared the effects of *TET1* and *DNMT3a* knockdown in ferroptosis. Preliminary data indicate that *DNMT3a* knockdown also enhances ferroptosis (see data below), albeit to a lesser extent than *TET1* knockdown (comparing with Supplementary Figs. 1l, m). A brief discussion has been added into Discussion, Page 18, lines 471-473. Given the focus of this manuscript, we have excluded the findings about *DNMT3a* from the main figures, as an in-depth discussion of another epigenetic regulator would exceed the current scope. However, this observation merits further investigation in future studies.

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Point-to-point details are listed below.

Comment 1 from Reviewer 2: *Figure 1a. TET1 was identified through RNA-seq using the Kasumi-1 cell line compared to the MV4;11 cell line. However, different cell lines, particularly THP1, were used in various downstream experiments. Including Kasumi-1 and MV4;11 as positive and negative controls, respectively, throughout the study would be beneficial.*

RESPONSE: We thank Reviewer for the suggestion. To keep consistency, we have included MV4;11 (ferroptosis-sensitive) and Kasumi-1 (ferroptosis-insensitive) as a paired set of model systems throughout the study, and have replaced most of the previous THP1-based figures accordingly. The new results are shown as Figs. 2n-o, 4a, 4c, 4e, 4g, 5a-b, 5e-f, 5h-i, 5l-n and Supplementary Figs. 2h-i.

Comment 2 from Reviewer 2: *Figure 1c:*

RESPONSE: We appreciate all the questions and concerns below regarding TET1 expression and function. Please see responses to each sub-question below.

1. How were basal expression levels of TET1 measured in 30 samples? qPCR (?): How many biological replicates? P-value? How about the TET1 protein levels?

–In Fig. 1c, the expression levels of *TET1* were determined with Q-RT-PCR. Each sample was tested for at least three independent times. Biological replicates have been included in figure legends, and error bars are shown on the figure. We further detected TET1 protein levels of the 33 (previous 30)-sample cohort with Western blotting, confirming similar positive correlation pattern between TET1 basal levels and cell viability after RSL3 treatment. Results are shown as Supplementary Fig. 1i.

2. TET1 showed extremely low expression in MV4;11, suggesting that MV4;11 might be independent of TET1. However, in Supplementary Figure 1J, TET1 knockdown by shRNAs (~50% knockdown efficiency) significantly reduced the cell viability of MV4;11. There is an inconsistent observation between TET1 expression and function.

–In MV4;11 cells, *TET1* expression is relatively low compared to ferroptosis-resistant cells such as Kasumi-1. However, both Q-PCR (lg-transformed) and Western blot analyses (Fig. 1c and Supplementary Fig. 1i) confirmed detectable *TET1*/TET1

expression in MV4;11, indicating that these cells are not *TET1* deficient. Importantly, gene expression levels do not always correlate directly with functional significance. Numerous genes with minimal expression have been demonstrated to play essential roles in cellular processes. For instance, key transcription factors like TP53 are often expressed at low basal levels unless activated, yet they critically regulate transcriptional programs (*Jordan et al., Proc Natl Acad Sci USA, 2012*). Similarly, pluripotency factors such as Nanog, Oct4, and Sox2 can exert profound effects on cell fate determination even at low expression levels (*Navarro et al., EMBO J, 2012; Jia et al., Theranostics, 2019; Baruah et al., Arterioscler Thromb Vasc Biol, 2020; Chen et al., Oncogene, 2025*). This principle extends to low-copy-number genes and non-coding RNAs (*Urick et al., Transpational Advances in Gynecologic Cancers, 2017*). Thus, the observed *TET1* expression in MV4;11 is not inconsistent with its functional role in ferroptosis regulation.

3. It is unclear how AML subtypes and driver mutations are related to TET1-mediated ferroptosis resistance.

–In order to better clarify the relationships between AML subtypes and TET1-mediated ferroptosis resistance, we have incorporated multiple patient-derived xenograft models and primary cells from AML patients. These primary AML samples encompass a wide range of AML karyotypes and subtypes, including those with *MLL*-rearrangements, *FLT3*-ITD, *DNMT3a, IDH1, WT1* or *NPM1* mutation(s), etc., covering French-American-British (FAB) classification subtypes from M2a to M5 (see Supplementary Table 2). Knockdown of *TET1* or TET1 inhibitor treatment showed ferroptosis sensitizing effect in various human primary AML cells *in vitro* (Figs. 6f, g and Supplementary Fig. 1p). PDX models were employed to test the therapeutic potential of the combinational therapies targeting the TET1 regulatory axis *in vivo* (Fig. 6h). Primary BM cells from four individual AML patients, including three RSL3-resistant (i.e., AML4/t(4;11), AML6/*FLT3*-ITD^{+}*NPM1*^{mut}*IDH1*^{mut} and AML7/t(9;11)) and one sensitive (AML3/ *FLT3*-ITD^{+}*NPM1*^{mut}*DNMT3a*^{mut}) patients, were used as donor cells. 100 days post xeno-BMTs, the BM engraftment and the infiltration of AML cells into peripheral blood were more dramatically suppressed by combined treatment with RSL3 and Bobcat339/BSO/DAHP than RSL3 treatment alone (Fig. 6i-l and Supplementary Fig. 6h-k). Results from these PDX models (*in vivo*) and primary AML cells collected from patients (*in vitro*) suggest the ferroptosis controlling effect of TET1 likely exists in many different subtypes of AML. TET1-mediated ferroptosis resistance seems to be a broad phenomenon in various AMLs, or even other cancers (see Figs. 1c, 1j-k and Supplementary Figs. 1a-d).

Interestingly, our findings demonstrate that *MLL*-rearranged AML exhibits variable sensitivity to ferroptosis, which can be either high (as in AML2) or low (as in

AML5/7). This difference appears to be more associated with the expression level of *TET1* in each individual patient (see Fig. 1c and Supplementary Figs. 1a-d).

4. Low TET1 was observed in MLL-AF9/MLL-AF4 cell line. MOLM13 (MLL-AF9) and MV4;11 (MLL-AF4) exhibited minimal TET1 expression and showed high sensitivity to the GPX4 inhibitor RSL3 (ferroptosis inducer). THP1 (MLL-AF9) has low TET1 but is RSL3-resistant. These observations are contrast with primary AML data, where genome-wide studies consistently report TET1 upregulation in primary MLL-rearranged AML, and TET1 cooperates with MLL fusions to drive leukemogenesis.

–According to the classification criteria outlined in Fig. 1c and Supplementary Figs. 1c-d, both MV4;11 and THP1 are ferroptosis-sensitive cell lines with relatively low *TET1* expression levels. We further validated the expression and functional relevance of *TET1* in multiple primary AML patient-derived cell samples, including 4 with *MLL*-rearrangements. As a matter of fact, when all tested *MLL*-rearranged AML samples were considered together, the average expression level of *TET1* was comparatively higher than that in non-*MLL*-rearranged AML samples (see data shown below), consistent with our previous report (*Huang et al., Proc Natl Acad Sci USA, 2013*). Moreover, the expression levels of *TET1* directly correlated with ferroptosis sensitivity. Results are included in the new Fig. 1c, and Figs. 6f-l.

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The key inconsistencies of TET1 expression between human cell lines and primary MLL AML implicate cell line limitations. Established AML cell lines (e.g., MOLM13, THP1) often lose epigenetic heterogeneity seen in primary samples, potentially explaining aberrant TET1 downregulation despite MLL rearrangements. Ferroptosis sensitivity in these lines may reflect compensatory pathways (e.g., low GSH synthesis) unrelated to TET1 status. Primary MLL-rearranged AML relies on TET1-mediated epigenetic regulation (e.g., 5hmC modification at oncogenic loci). Cell lines may bypass this dependency through acquired mutations or culture adaptations. The questions are: do primary MLL-rearranged AML cells with high TET1 exhibit ferroptosis resistance due to the role of TET1 in glutathione metabolism (via GCLC)? Conversely, does TET1-low status (e.g., TET1 knockdown) correlate with RSL3 sensitivity in primary AML patient-derived xenografts?

–In primary AML cells from AML patients, spanning both ferroptosis-sensitive and -resistant subtypes, the basal level of GSH in those with relatively higher *TET1* expression (*TET1*^{high}) was significantly higher than in *TET1*^{low} cells (Supplementary Fig. 3a). In these primary AML cells, *TET1* knockdown significantly sensitized cells to ferroptosis, while *TET1* overexpression conferred resistance (Supplementary Fig. 1p). With patient-derived xenograft models (Fig. 6h), we tested the effect of TET1 suppression on ferroptosis sensitivity *in vivo*. Primary BM cells from four individual AML patients, including one sensitive (AML3/*FLT3*-ITD^{+}*NPM1*^{mut}*DNMT3a*^{mut}, *TET1*^{low}) and three RSL3-resistant (i.e., AML4/t(4;11), AML6/*FLT3*-ITD^{+}*NPM1*^{mut}*IDH1*^{mut} and AML7/t(9;11), *TET1*^{high}) patients, were used as donor cells. 100 days post xeno-BMTs, the BM engraftment and the infiltration of AML cells into peripheral blood were more dramatically suppressed by combined treatment with RSL3 and Bobcat339 than RSL3 treatment alone (Fig. 6i-l and Supplementary Fig. 6h-k). The above results validated the correlation between TET1 expression/activity and ferroptosis sensitivity in primary AML models.
In general, results of primary AML cells and PDX models verify the expression variation of *TET1* in AMLs, including *MLL*-rearranged AMLs, as well as the correlation between TET1 expression/function and ferroptosis resistance.

Comment 3 from Reviewer 2: *Figure 1f,g: These figures illustrate the in vitro treatment of mouse AML cells, derived from Tet-/- mice, with RSL3. It is important to clearly state this in the manuscript text to avoid any confusion with in vivo treatment. Additionally, would the authors consider conducting in vivo treatment of AML Tet-/- mice with RSL3?*
RESPONSE: We thank Reviewer for this suggestion. Figs. 1f and g are *ex vivo* studies, showing effects of *Tet1* deficiency on RSL3 sensitivity *in vitro*. The description has been revised on Page 8, line 180 and in corresponding figure legends accordingly. To better clarify how *Tet1* knockout affects RSL3 response *in vivo*, BMT assays using *Tet1* inducible knockout AML cells as donor cells were conducted. Consistent with *in vitro* results, *in vivo* studies showed a better effect of RSL3 in prolonging overall survival and restricting AML cell engraftment of recipient mice transplanted with *Tet1* deficient *MA9*-AML cells compared to wild-type (WT) *MA9*-AML cells (Supplementary Fig. 1q, r). These results further validated the ferroptosis sensitivity determining effect of *Tet1 in vivo*.

Comment 4 from Reviewer 2: *Figure 2d,e: Would knockdown and overexpression of TET1 change GCLC protein levels?*

RESPONSE: We thank Reviewer for the question. Knockdown of *TET1* reduced *GCLC*/GCLC expression, while overexpression of *TET1* significantly increased *GCLC*/GCLC expression, at both transcription (as determined through Q-RT-PCR) and protein (as detected with Western blotting) levels. Results are shown as the new Figs. 2d and e.

Comment 5 from Reviewer 2: *Iron levels are a crucial determinant of ferroptosis. What are the iron levels in human and mouse cells treated with RSL3?*
RESPONSE: A good question it is. In human AML cell lines and mouse AML cells, RSL3 treatment increased cellular labile iron levels, particularly Fe^{2+} (Supplementary Fig. 1e), in consistence with previous reports (*Wang et al., Redox Biol, 2024; Sui et al., Front Pharmacol, 2018*).

Comment 6 from Reviewer 2: *How were the RNA-seq data analyzed, and what cutoff criteria were used?*
RESPONSE: RNA-seq data were analyzed using FPKM (Fragments Per Kilobase of transcript per Million mapped reads) values. Differential gene expression analysis was performed between Kasumi-1 and MV4;11 cell lines, with significantly differentially expressed genes identified using the following cutoff criteria: absolute log2(fold change) ≥ 1 and adjusted P-value ≤ 0.05. This analysis revealed 1,516 upregulated genes in Kasumi-1 compared to MV4;11. Subsequently, Gene Set Enrichment Analysis (GSEA) was performed on these 1,516 upregulated genes to identify enriched biological pathways and functional categories. The above information has been included into figure legends related to Fig. 1a.

Comment 7 from Reviewer 2: *If TET1 controls cancer cell susceptibility to ferroptosis in both GPX4-dependent and -independent mechanisms, which pathway is predominant and how do these two pathways interact? If one pathway is inhibited, would the other compensate?*
RESPONSE: We thank Reviewer for the very good question! According to our results (Figs. 4c-d) and others' reports (*Liang et al., Cell, 2023*), *GPX4* knockout more often results in almost complete blockade of cell survival, especially in ferroptosis sensitive cells like MV4;11, while *GCH1* knockout only partially suppresses cell viability (*Soula et al., Nat Chem Biol, 2020*). Additional experiments further confirmed that genetic ablation of *GPX4* resulted in a more pronounced suppression in cell viability compared to *GCH1* knockout, indicating the predominant role of GPX4 in AML ferroptosis surveillance (Supplementary Fig. 5j). Intriguingly, we observed a reciprocal regulatory relationship between GCH1 and GPX4: Genetic

inhibition of *GPX4* triggered compensatory BH4 accumulation through GCH1 activation, while *GCH1* ablation conversely enhanced GPX4 activity (Supplementary Figs. 5h, i). This feedback regulation suggests an intricate crosstalk between these two major ferroptosis defense systems, potentially maintaining redox homeostasis in cancer cells under certain conditions.

Comment 8 from Reviewer 2: *Most experiments have been conducted using human cell lines, which do not fully recapitulate patient conditions. It is crucial to determine whether and how TET1-mediated ferroptosis resistance applies to patient-derived xenograft (PDX) models from primary AML samples and how TET1-mediated ferroptosis resistance is linked to AML subtypes and their driver mutations.*
RESPONSE: We thank Reviewer for the constructive suggestion! According to Reviewer's suggestion, we have incorporated multiple patient-derived xenograft models and primary cells from AML patients. These primary AML samples encompass a wide range of AML karyotypes and subtypes, including those with *MLL*-rearrangements, *FLT3*-ITD, *DNMT3a, IDH1, WT1* or *NPM1* mutation(s), etc., covering FAB classification subtypes from M2a to M5 (see Supplementary Table 2). Knockdown of *TET1* or TET1 inhibitor treatment showed ferroptosis sensitizing effect in various human primary AML cells *in vitro* (Figs. 6f, g and Supplementary Fig. 1p). PDX models were employed to test the therapeutic potential of the combinational therapies targeting the TET1 regulatory axis *in vivo* (Fig. 6h). Primary BM cells from four individual AML patients, including three RSL3-resistant (i.e., AML4/t(4;11), AML6/*FLT3*-ITD^{+}*NPM1*^{mut}*IDH1*^{mut} and AML7/t(9;11)) and one sensitive (AML3/ *FLT3*-ITD^{+}*NPM1*^{mut}*DNMT3a*^{mut}) patients, were used as donor cells. 100 days post xeno-BMTs, the BM engraftment and the infiltration of AML cells into peripheral blood were more dramatically suppressed by combined treatment with RSL3 and Bobcat339/BSO/DAHP than RSL3 treatment alone (Fig. 6i-l and Supplementary Fig. 6h-k). Results from these PDX models (*in vivo*) and primary AML cells collected from patients (*in vitro*) suggest the ferroptosis controlling effect of TET1 likely exists in many different subtypes of AML. TET1-mediated ferroptosis resistance seems to be a broad phenomenon in various AMLs, or even other cancers (see Figs. 1c, 1j-k and Supplementary Figs. 1a-d).

Minor Comments from Reviewer 2:

1. *Please state the number of biological replicates performed across all experiments, as well as the method used to calculate the p-value. Some statistical analyses appear to be incorrect. For example, Figures 1f, 1g, 1j, and 1k might require two-way ANOVA, while Figures 1e, 1h, and 1i might require one-way ANOVA, and so on.*

RESPONSE: We thank Reviewer for pointing out the inappropriate statistical approaches. The statistical methods of Figs. 1f, 1g, 1j and 1k have been changed to two-way ANOVA. The statistical approaches of Fig. 1e, 1h and 1i have been changed into one-way ANOVA. The numbers of biological replicates have been labeled in all figure legends across the manuscript. All the other remaining statistical errors have been carefully corrected.

2. *Figure 1j is difficult to read and needs to be presented more clearly. Additionally, it lacks statistical analysis.*
RESPONSE: Figs. 1j and 1k have been split into isolated figure panels to clearly show results of each cell line. Statistical analyses have been described in figure legends.

3. Responses to the comments of Reviewer 3 (co-review with Reviewer 1)**:**
General Comments from Reviewer 3**:** *I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.*
RESPONSE: We thank Reviewer for co-reviewing this manuscript. Please see "1. Responses to the comments of Reviewer 1" for detailed responses.

4. Responses to the comments of Reviewer 4:
General Comments from Reviewer 4**:** *This manuscript by Yang et al. presents a compelling and well-executed study identifying TET1 as a central regulator of ferroptosis resistance in cancer, with a particular focus on acute myeloid leukemia (AML). The authors demonstrate that TET1 modulates both GPX4-dependent and -independent ferroptosis surveillance pathways by transcriptionally regulating GCLC and GCH1 via 5hmC-mediated epigenetic remodeling and NF-κB signaling. The use of shRNA knockdowns, overexpression constructs, ChIP-seq, 5hmC-seq, RNA-seq, in vivo AML mouse models, and human AML samples provides robust experimental support. This study brings novel insights into the epigenetic control of ferroptosis and highlights TET1 as a potential therapeutic target in ferroptosis-resistant AML. However, several critical points require clarification and additional data to ensure the broader validity and mechanistic precision of the conclusions.*
RESPONSE: We appreciate Reviewer's pertinent comments and positive appraisals. To ensure the broad validity and strengthen the mechanism study, we conducted additional experiments and made substantial revision to the manuscript. Please see point-to-point responses below.

Comment 1 from Reviewer 4: *The manuscript title refers to TET1 as a "master regulator of ferroptosis surveillance in cancer." However, the study is almost exclusively focused on acute myeloid leukemia (AML), as evidenced by the use of human AML cell lines, murine AML models (MA9 and AE9a), and AML patient samples. TET1 has been shown to play divergent roles in hematological malignancies—tumor-suppressive in B-cell acute lymphoblastic leukemia (B-ALL) and oncogenic in T-cell ALL (T-ALL). Thus, the generalization to "cancer" may overstate the findings. A more accurate title, such as "TET1 as a master regulator of ferroptosis surveillance in AML", would better reflect the specificity and experimental scope of the study.*

RESPONSE: We appreciate Reviewer's suggestion. The manuscript title has been changed into "TET1 as a master regulator controlling both GPX4-dependent and -independent ferroptosis surveillance in AML" according to Reviewer's comments.

Comment 2 from Reviewer 4: *AML is sustained by a subset of self-renewing leukemic initiating cells (LICs), which are thought to mediate therapy resistance and disease relapse. While the study thoroughly characterizes TET1's role in bulk AML populations, it remains unclear whether TET1 regulates ferroptotic susceptibility in LICs. Investigating this would be of high translational relevance. Specifically, evaluating LIC frequency and function after co-treatment with RSL3 and TET1 inhibitors (e.g., Bobcat339, BSO, DAHP) in LIC-enriched compartments or serial transplantation assays could offer valuable insights.*

RESPONSE: We sincerely appreciate Reviewer's good suggestion. In order to investigate the role of *TET1* in LIC ferroptosis response, mouse AML model was constructed through BMT, and LIC enrichments were tested in BM of AML recipient mice. Flow cytometry analysis of *MA9*-AML bearing recipient mice demonstrated that single-agent treatment with either Bobcat339, BSO or DAHP significantly reduced the LIC-enriched c-kit$^+$-Mac-1$^+$ population. Notably, combination therapy with RSL3 plus Bobcat339, BSO or DAHP resulted in a more profound decrease of LICs (Supplementary Fig. 6g), suggesting that targeting TET1-dependent ferroptosis surveillance systems may provide superior therapeutic efficacy against LICs.

Comment 3 from Reviewer 4: *According to the methods, the authors overexpressed the catalytic domain (CD) of TET1, which lacks the N-terminal CXXC zinc finger DNA-binding domain essential for site-specific chromatin targeting. While the catalytic domain can mediate hydroxymethylation, it may not fully recapitulate the genomic localization or gene regulatory functions of endogenous full-length TET1.*

Therefore, conclusions regarding direct transcriptional activation of target genes (e.g., GCLC) would be more robust if validated by overexpressing full-length TET1 and performing ChIP-qPCR or luciferase reporter assays to confirm target engagement.

RESPONSE: We appreciate Reviewer's comments. Following Reviewer's suggestions, we constructed the expression plasmids encoding full-length *TET1* (*TET1*-FL), and conducted ChIP-Q-PCR and luciferase reporter assays. Results of *TET1*-FL are consistent with those of *TET1*-CD. ChIP-Q-PCR results verified the association between *TET1*-FL and the promoter region of *GCLC* (Supplementary Fig. 2f). Luciferase reporter assays confirmed the transcriptional activation of *GCLC* by *TET1*-FL (Supplementary Fig. 2g). The above results have been included as new Supplementary Figs. 2f and g.

Comment 4 from Reviewer 4: *The manuscript establishes a link between TET1 and ferroptosis-related genes (e.g., GCLC, GCH1) in cell lines. To enhance translational relevance, the authors should extend this analysis to primary patient datasets, such as TCGA AML and solid tumor cohorts, using tools like cBioPortal. Assessing whether TET1 expression correlates with GCLC and GCH1 expression in patient-derived transcriptomic data would validate these findings in clinically relevant settings.*

RESPONSE: We thank Reviewer for the constructive suggestion. Through additional bioinformatics analysis, we found significant positive correlation between the expression levels of *TET1* and *GCLC*, in both TCGA (n=150) and GSE30285 (n=93) AML datasets (Supplementary Fig. 2b). Although we did not find significant positive correlation in expression of *TET1* and *GCH1* in the above datasets (data not shown), a significant positive correlation between TET1 and GCLC protein levels (Pearson r=0.627, P<0.001), and a positive trend between TET1 and GCH1 expression (Pearson r=0.268, P=0.131) were observed in our 33-sample cancer cell cohort with WB and statistical analysis (Supplementary Fig. 4s). Notably, no significant association was detected between TET1 and FSP1 levels (Pearson r=-0.138, P=0.307) (Supplementary Fig. 4s). The positive correlation between *TET1* and *GCLC* or *GCH1* gene expression levels was further confirmed in the 33-sample cancer cell cohort with Q-RT-PCR (Supplementary Fig. 2c and 5a).

Comment 5 from Reviewer 4: *TET2 and TET3 share overlapping enzymatic functions with TET1 in catalyzing 5mC to 5hmC conversion and are often co-expressed in hematopoietic cells. It is plausible that knockdown or overexpression of TET1 could affect the expression or activity of TET2/TET3, which might confound interpretation of TET1-specific effects. The authors should therefore evaluate TET2 and TET3*

expression in TET1 knockdown and overexpression models to exclude functional redundancy or compensatory regulation.

RESPONSE: Very good suggestion. In multiple AML cell lines, i.e., MV4;11, THP-1 and Kasumi-1, as well as solid tumor cell line A375, knockdown of *TET1* by use of shRNA or overexpression of *TET1* results in no obvious alterations in the levels of *TET2* and *TET3* (see Supplementary Figs. 1l-o, and 1u-w), underscoring the specific role of TET1 in ferroptosis regulation.

Comment 6 from Reviewer 4: *The manuscript reports correlations between TET1 expression levels and ferroptosis sensitivity in various cancer cell lines. However, the methodology for quantifying TET1 expression is not clearly stated. The authors should clarify whether RNA-seq or qRT-PCR was used. If RNA-seq was employed, it is important to report the normalization metric (e.g., TPM, FPKM), source of the dataset (e.g., in-house sequencing, CCLE, TCGA), and the statistical method (e.g., Pearson/Spearman correlation) used in the analysis.*

RESPONSE: For correlation analysis between *TET1* expression and ferroptosis sensitivity, expression levels of *TET1* were detected with Q-RT-PCR and were normalized to *TET1* basal level of Daudi cells. Pearson analysis of lg-transformed relative *TET1* expression levels and relative cell viability was performed. A description has been added into the corresponding figure legends related to Fig. 1c.

Comment 7 from Reviewer 4: *To increase confidence in the RNA-seq-derived expression data, the authors should validate TET1, GCLC, and GCH1 expression levels in a representative panel of ferroptosis-sensitive and -resistant cell lines using qRT-PCR. This step is standard in transcriptomic studies and serves to confirm the accuracy and reproducibility of computational findings.*

RESPONSE: We thank Reviewer for the comments! Previous Fig. 1c showed in our 33-cancer sample cohort the correlation between relative cell viability determined with MTT and *TET1* expression levels detected through Q-RT-PCR, instead of RNA-seq-derived readouts. To avoid confusion, independently triplicated results are now included and error bars are shown. Corresponding figure legends have been revised accordingly. We detected the gene expression levels of *GCLC* and *GCH1* in the same 33-sample cohort with Q-RT-PCR as well. Pearson correlation analyses between *GCLC* or *GCH1* expression levels and *TET1* gene levels (all Q-RT-PCR results) are also included as the new Supplementary Figs. 2c and 5a.

Comment 8 from Reviewer 4: *Transcript abundance does not always correlate with protein levels due to post-transcriptional regulation. The authors should therefore*

confirm TET1 protein expression using Western blotting across key cell lines used in the study. This would reinforce the mechanistic link between TET1 levels and ferroptosis resistance and clarify any post-transcriptional modulation of TET1.
RESPONSE: We thank Reviewer for the suggestion! TET1 protein expression levels were detected with Western blotting. Results are shown as part of the new Figs. 1f-g, 2d-e and Supplementary Fig. 1i.

Minor Comments from Reviewer 4:
1. In lines 286–287, the authors state that "ChIP-q-PCR analysis verified the association between TET2 and the promoter region of NFKB2 (Fig. 5c)," which appears to be a typographical error. Based on the context of the study and the figure legend, the ChIP assay was performed for TET1, not TET2. The authors should correct this to maintain consistency and avoid confusion.
RESPONSE: We thank Reviewer for his/her careful going over. This typo has been corrected.

5. Responses to the comments of Reviewer 5:
General Comments from Reviewer 5**:** *In the present study, the authors demonstrate that TET1 enhances resistance to ferroptosis in cancer cells by improving antioxidant defense mechanisms. The knockdown of TET1 increases sensitivity to ferroptosis inducers, such as RSL3, in both ferroptosis-sensitive and -resistant cancer cells, which leads to elevated levels of reactive oxygen species (ROS) and lipid peroxidation. These effects are reversible with the application of ferroptosis inhibitors. Mechanistically, TET1 transcriptionally activates GCLC, a critical enzyme in glutathione (GSH) synthesis, through 5hmC modifications and promoter binding, thus increasing GSH production and alleviating ferroptotic stress. Additionally, TET1 promotes the synthesis of non-canonical γ-glutamyl-peptides under conditions of cystine deprivation, which supports glutamate scavenging. Importantly, the protective role of TET1 extends beyond dependence on GPX4, indicating its involvement in alternative ferroptosis defense mechanisms, such as the GCH1–BH4 pathway. TET1 regulates the expression of GCH1 via the activation of NFκB signaling, particularly through NFKB2, thereby adding another dimension to its role in resistance to ferroptosis. The TET1/GCLC and TET1/NFκB/GCH1 pathways collectively establish TET1 as a potential regulator of cancer cell susceptibility to ferroptosis. The manuscript is overall interesting.*
RESPONSE: We appreciate Reviewer's comments and positive appraisals. Please see point-to-point responses below.

Comment 1 from Reviewer 5: *Testing GCLC expression and GSH/GSSG levels in NFE2L2 knockout/TET1-high cancer cells could help demonstrate the impact of TET1 in regulating GCLC.*

RESPONSE: We appreciate Reviewer's constructive suggestion. *NFE2L2* knockout suppressed *GCLC* expression and GSH level (Supplementary Fig. 2p and 3b), consistent with previous reports (*Tang et al., Cancer Drug Resist, 2024; Kang et al., Cell Metab, 2021; Tonelli et al., Antioxid Redox Signal, 2018*). Interestingly, overexpression of *TET1* partially restored *GCLC* expression (Supplementary Fig. 2o, p) and GSH level (Supplementary Fig. 3b) in *NFE2L2*-deficient cells, suggesting the potential crosstalk between of TET1 and the known NFE2L2/*GCLC* axis.

Comment 2 from Reviewer 5: *FSP1 is recognized as one of the major contributors to ferroptosis defense mechanisms, functioning independently of glutathione. In this context, do the TET1-high cancer cell lines shown in Figure 1c rely on TET1 activity to compensate for the lower expression of FSP1? It would be helpful to present the trends in protein expression of FSP1, TET1, and GCH1 across the cancer cell lines used in the study (primarily AML).*

RESPONSE: We thank Reviewer for the suggestion. The protein expression levels of TET1, GCLC, FSP1 and GCH1 in a 33-cancer sample cohort were detected with Western blot. The quantification results revealed a statistically significant positive correlation between TET1 and GCLC protein levels (Pearson $r=0.627$, $P<0.001$), and a positive trend was observed between TET1 and GCH1 expression (Pearson $r=0.268$, $P=0.131$). Notably, no significant association was detected between TET1 and FSP1 levels (Pearson $r=-0.183$, $P=0.307$). The above results are shown as the new Supplementary Fig. 4s.

Comment 3 from Reviewer 5: *Regarding the investigation of the TET1/GCLC axis in GSH and γ-glutamyl-peptide synthesis (Figure 3), the readout should include not only glutathione levels but also cell viability, given the effects of glutathione depletion. I suggest extending the cystine starvation period beyond 12 hours in TET1 knockout and overexpression cells and assessing whether TET1-OE cells show improved survival due to higher γ-glutamyl-peptide levels.*

RESPONSE: We thank Reviewer for this suggestion. In Mx1-Cre$^{+/-}$*Tet1*$^{flox/flox}$ *MA9*-AML mouse cells, induced depletion of *Tet1* exacerbated the suppression of cell viability resulted from cystine withdrawal, whereas *Tet1* overexpression effectively rescued this phenotype (Supplementary Fig. 3f). Meanwhile, *Tet1* knockout reduced γ-glutamyl-peptide synthesis induced by cystine deprivation, whereas restore of *Tet1*

expression in the above cells could reverse such reduction (Supplementary Fig. 3g), suggesting the potential association between *Tet1* levels, γ-glutamyl-peptide levels and cell viability. The above results are shown as Supplementary Figs. 3f-g.

Comment 4 from Reviewer 5: *To demonstrate that the upregulation of GCH1 activity induced by TET1 and NF-κB protects cells from lipid peroxidation, measuring tetrahydrobiopterin levels while modulating TET1 and NF-κB expression would be beneficial.*

RESPONSE: We appreciate Reviewer's good suggestion. Ectopic expression of either *TET1* or *NFKB2* led to a significant elevation of intracellular BH4 levels and a decrease of RSL3-induced ROS accumulation (Fig. 5p and Supplementary Fig. 5f). Conversely, knockdown of *TET1* or *NFKB2* resulted in a marked reduction of BH4 production and an increase of RSL3-induced ROS accumulation (Fig. 5q and Supplementary Fig. 5g). Our findings establish a previously unidentified regulatory axis wherein both TET1 and NFKB2 critically modulate GCH1 expression and/or function, thereby unveiling the existence of the TET1/NFκB signaling/GCH1 axis in cancer ferroptosis regulation.

Specific points from Reviewer 5:

1. *Line 74-76: The genetic regulation of GSH and γ-glutamyl-peptides biosynthesis has been investigated. I would suggest to mention that NRF2 transcribes GCLC, GCLM, GSR1, xCT (PMID: 28899199) and also GCLC regulates γ-glutamyl-peptides biosynthesis (PMID: 33357455).*

RESPONSE: The suggested references have been cited on lines 81 and 237, respectively.

2. *Line 80: dihydroorotate dehydrogenase (FSP1) ferroptosis suppressor protein 1 (FSP1)*

RESPONSE: This typo has been corrected.

3. *Line 118-119: To emphasize the correlation between TET1 expression and sensitivity to GPX4 inhibition, it would be better to explain that both RSL3 and ML210 inhibit the enzymatic activity of GPX4, thereby inducing ferroptosis.*

RESPONSE: The above description has been added and reference papers have been cited on lines 137-138.

4. *Line 696-698: 200 mM cystine supplementation in the cystine/cysteine/methionine/glutamine-free RPMI-1640 seems to be 1000 times higher*

concentration than in a standard culture medium condition. Is it the correct concentration? Additionally, please confirm the methionine concentration in the medium used for the experiment (100 mM).

RESPONSE: We sincerely appreciate Reviewer's careful review of our manuscript. We acknowledge the typographical errors in the originally reported concentrations and would like to clarify that the correct concentrations are **200 µM for cystine** and **100 µM for methionine** (not mM, as previously stated). We sincerely apologize for any confusion these inaccuracies may have caused and have corrected them in the revised manuscript (see lines 903-904).

5. *Western blots are missing molecular weight mark.*

RESPONSE: According to reviewers' comments, a number of Western blot results are included in the manuscript as Figs. 1f-g, 2d-e, 4a-b, Supplementary Figs. 1i, 3i, 4o and 4s. Molecular weight marks are labeled in the above figures, and can be seen on original slide images deposited online.

<u>Comments on Figures from Reviewer 5:</u>

1. *Figure 1*

- Has the knockdown and knockout of TET1 in the cells been confirmed through protein or gene expression analysis? It would be beneficial to present this alongside the phenotypic analysis.

RESPONSE: The knockdown and knockout efficiency of TET1/*TET1* have been confirmed through WB and/or Q-RT-PCR. Results are shown alongside the previous Figs. 1f, 1g, and Supplementary Figs. 1l-o.

2. *Figure 3*

- q) System X⁻ System xc-.

- q) The arrow linking glutamate and ferroptosis could result in a misinterpretation that glutamate accumulation is the direct cause of ferroptosis.

RESPONSE: Fig. 3p (previously Fig. 3q) has been revised following Reviewer's suggestions.

3. *Figure 5*

p) System X⁻ → System xc-.

p) A line is missing to connect glycine to the glutathione synthesis pathway (as shown in Figure 3-q).

RESPONSE: Fig. 5r (previous Fig. 5p) has been revised following Reviewer's suggestions.

Minor points from Reviewer 5:

- *Including "TET1-HxD: TET1 catalytically dead mutant" in the Abbreviation section is better.*
- *This study's main finding is that TET1 contributes to the defense against ferroptosis by regulating the expression of GCLC and GCH1. Incorporating these key points could make the title of the manuscript more specific.*

RESPONSE: The abbreviation has been added and the title has been revised according to the suggestions.

Responses to Reviewers' Comments:
Manuscript ID: NCOMMS-25-18361
Manuscript Title:
TET1 as a master regulator controlling both GPX4-dependent and -independent ferroptosis surveillance in AML

SUMMARY: We are very grateful to all the reviewers for their positive comments and insightful suggestions. We have thoroughly addressed all remaining comments by incorporating new experimental results and implementing necessary revisions throughout the manuscript. All the text in the manuscript that we changed or added during the revision is marked in red. Deleted text has been removed entirely from the file. Our detailed responses are described below. We hope that the version after second revision would better meet the requirements of publication in *Nature Communications*.

Reviewers #1, #3 and #7:

Response: We would like to express our sincere gratitude to the reviewers for their positive comments and are pleased to finalize the manuscript for publication.

Reviewer #2:

General Comments: *The reviewer acknowledges the authors' efforts in addressing most of the questions raised. Nevertheless, two questions remain unresolved and require further clarification:*
Response: We sincerely appreciate Reviewer's comments and constructive suggestions. To address these points, we have conducted additional experiments, incorporated new markers and included corresponding figures. Our point-by-point responses to the two questions are detailed below.

Comment 1: *The legend of Suppl Fig. 1i states: "Expression levels of TET1 were detected with Western blot (WB) and data were 31 normalized to basal TET1 level of Kasumi-1 cells. (red: ferroptosis-sensitive, blue: ferroptosis-insensitive)."*
- Please specify how TET1 protein levels were determined. For better interpretability, please quantify the western blot bands and displaying the relative expression values above each TET1 band in the figure.
Response: Pursuant to Reviewer's suggestion, the Western blot results have been quantified, with the relative expression values now indicated below the respective bands (Supplementary Fig. 1i, middle panels). The quantification was performed as follows: Band intensity was analyzed using Gel-Pro Analyzer software. The background was subtracted for each band, and the values were normalized to the internal control (GAPDH). The normalized values were then expressed relative to the basal TET1 level of Kasumi-1 cells. Average values from at least three independent experiments are included in statistical analysis. This protein quantification procedure has been thoroughly described in the updated legend for Supplementary Fig. 1i.
- Based on the current WB data, TET1 protein expression does not appear to consistently correlate with ferroptosis sensitivity. For example, Kasumi-1 (blue; ferroptosis-insensitive) shows relatively high TET1

expression compared to MV4;11 (red; ferroptosis-sensitive). Conversely, other ferroptosis-insensitive cell lines such as MIA PaCa-2, ASPC-1, HepG2, and NB4 exhibit low TET1 levels, similar to or even lower than those in MV4;11. Likewise, ferroptosis-sensitive cell lines like H292 and THP1 display high TET1 expression comparable to that of Kasumi-1.

Response: We appreciate Reviewer's meticulous attention to detail. Indeed, we note that a small subset of ferroptosis-resistant cells exhibits lower TET1 levels than the average across all cancer cells tested. Nevertheless, our statistical analysis confirms that the average TET1 level in ferroptosis-resistant cells remains significantly higher than that of their sensitive counterparts. This conclusion is further supported by a significant negative correlation between TET1 protein levels and RSL3 sensitivity across the entire set of 33 samples. To clearly present these findings, we have now included the statistical analysis of the Western blot results in Supplementary Fig. 1i (left and right panels), which illustrates both the correlation and the group comparison.

Comment 2: *For in vivo treatment of PDX models in Fig. 6h-l:*

- While CD33 is commonly used to identify AML blasts, it is also expressed on normal human myeloid cells. This overlap can lead to false positives when CD33 is used as a sole marker. To improve specificity, additional AML-associated surface markers should be considered, such as hCD45/mCD45, which were used in the CDX models shown in Suppl Fig. 6n and 6o.

Response: We thank Reviewer for the suggestion. As suggested, we have included human CD45 and mouse CD45 are included as a pair of secondary markers to assess AML cell engraftment in our PDX models. The corresponding flow cytometry data and statistical analysis are now presented in new Figs.6i and 6j. The results are consistent with our primary human CD33 data, which, for clarity, have been moved to Supplementary Figs. 6h-k.

- Could you provide data comparing spleen sizes between treated and control mice, as well as the extent of AML blast cell infiltration into spleen tissue? This would help assess treatment efficacy and disease burden.

Response: At 100 days post-xeno-BMTs, we collected and weighed the spleens from a cohort of recipient mice (n=5 per group). Despite comparison across all groups, no significant differences in spleen weight were observed (data shown below). Furthermore, no obvious $hCD45^+$ or $hCD33^+$ populations were detected in this organ (data not shown).

This absence of significant engraftment in the spleen may be attributed to two non-exclusive possibilities. **First,** as supported by the data in Figs. 6i and j, the tested AML cells (AML4 and AML7) may exhibit a tissue tropism that favors bone marrow engraftment over spleen infiltration, a phenomenon consistent with the known diversity in aggressiveness and tissue tropism of AML cells (*Saland E et al., Blood Cancer J, 2015*). **Second,** the 100-day time point may precede full leukemic outgrowth, as most mice had not reached endpoint criteria. It is plausible that overt spleen infiltration requires a longer latency than initial bone marrow engraftment.

UNPUBLISHED DATA FIGURE REDACTED

- Additionally, could the PDX models be used to investigate whether primary AML cells with high TET1 expression exhibit ferroptosis resistance, potentially due to TET1's role in regulating glutathione metabolism via GCLC?

Response: We appreciate Reviewer's question. To this end, we collected bone marrow cells from mice at 100 days post-xeno-BMT and isolated the hCD45$^+$ populations. Across the four AML patient-derived cell samples, a positive correlation was observed among *TET1* expression, *GCLC* expression and GSH levels. These parameters were, in turn, negatively correlated with sensitivity to RSL3 (Supplementary Fig. 6l), thereby providing further *in vivo* validation for the proposed TET1/GCLC/GSH axis.

Reviewers #5-#6:

General Comments: *Thank you for the opportunity to review the revised manuscript. I am very impressed by the authors' detailed responses and the significant effort they put into conducting additional experiments. I have reviewed the authors' responses and the revised manuscript, and my previous comments have been addressed as follows:*

Response: We are grateful for Reviewers' positive comments and are greatly inspired by their insightful hypothesis, particularly the one raised in Comment 1. Our point-by-point response is provided below.

Comment 1: *The restoration of GCLC expression and GSH levels by overexpressing TET1 in NFE2L2-KO cells suggests that GCLC might be transcribed by other transcription factors, and this process could be supported by TET1-mediated DNA demethylation of the GCLC promoter region. While the experiment you performed meets my initial suggestion, this new data raises an interesting follow-up question: is TET1's demethylation activity essential for GCLC transcription? This could be investigated by analyzing GCLC expression levels in TET1 knockdown cells treated with an NRF2 activator (e.g., KI696). However, this is a new area of inquiry that is not critical for the current manuscript and does not require new experiments.*

Response: This is an insightful question, and we thank Reviewers for it. In the present study, we demonstrate that TET1 transcriptionally activates *GCLC* through demethylation. It has been reported that *GCLC* expression is under sophisticated transcriptional control. While NFE2L2 serves as a core

transcription factor, other regulators—including NFκB, Sp-1, activator protein-1 (AP-1), metal response (MRE), and antioxidant response (ARE)/electrophile responsive (EpRE) elements, etc., —have also been shown to bind the promoter region of *GCLC*, and collectively regulate its transcription. For instance, in gastric cancer cells, actin-like protein 6A (ACTL6A) cooperates with NFE2L2 to promote *GCLC* expression and confer ferroptosis resistance. Here, we demonstrate that TET1 restores *GCLC* expression and partially rescues its function even under *NFE2L2*-deficient conditions. While it remains unclear whether TET1 is essential for NFE2L2-mediated transcriptional regulation, our findings indicate that TET1 may serve as a previously unrecognized key regulator of *GCLC*, acting at least in part independently of NFE2L2. These results further suggest potential crosstalk between TET1 and the established NFE2L2/GCLC axis, highlighting the need for further investigation into the precise role of TET1 within this axis, as well as in the broader transcriptional network governing *GCLC* expression. We have added this perspective to the Discussion (see Page 19).

Other comments:

Comment-2: The effort on expression analysis with patient-derived cancer samples clarified the positive correlation between TET1 and GCLC. I also appreciate that the authors clarified there is no strong correlation between TET1 and FSP1.

Comment-3: The new experiments in Supplementary Figures 3f and 3g directly address my previous question. The data clearly demonstrates that cell viability under cystine starvation depends on TET1 expression.

Comment-4: All the new experiments have excellently addressed the previous suggestions and have significantly improved the overall quality of the manuscript.

Specific and minor points: Upon reviewing the revised version of the manuscript, I find that the text, figures, schemes, and title are now clearly presented.

Overall, the authors have expanded on their findings with newly conducted experiments. Based on these revisions, I recommend acceptance for publication without further changes.

Response: We extend our sincere thanks to Reviewers for their valuable comments.