

Autophagy Regulates the Hepatic Acetylome Through Modulating Acetyl-CoA Metabolite

Corresponding Author: Dr Bilon Khambu

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

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

This study provides novel insights into the intersection between autophagy and post-translational modifications (PTMs) in the liver, with a particular focus on protein acetylation. While the role of acetylation in autophagy is well recognized, the reverse impact of autophagy on acetylation remains underexplored. The authors employ multiple models of autophagy modulation to demonstrate that impaired autophagy leads to global changes in PTMs, notably a marked reduction in protein acetylation linked to suppressed acetyl-CoA biosynthesis. They further identify NRF2 as a transcriptional repressor of key enzymes in the acetyl-CoA production pathway, positioning it as a central mediator of this metabolic regulation. These findings highlight a previously unappreciated role of autophagy in maintaining hepatic acetylation status through metabolic control, with significant implications for liver disease pathogenesis and therapy. However, there are several concerns that need to be addressed:

1. The relationship between acetylation and autophagy is well established, i. e., when acetylation decreased autophagy increased (PMID: 37224468). Previous studies also have shown that reduced cytosolic protein acetylation can activate autophagy, positioning acetylation as a nutrient-sensitive regulatory mechanism. Specifically, autophagy is inversely correlated with cytosolic acetyl-CoA levels, suggesting that fluctuations in acetyl-CoA availability modulate autophagic activity in response to cellular metabolic status (Eisenberg et al., 2014). Under nutrient-rich conditions, cytosolic accumulation of acetyl-CoA enhances protein acetylation, which in turn suppresses autophagy, ultimately contributing to impaired metabolic homeostasis (Schroeder et al., 2014). This suppression is mediated through acetylation of key autophagy-related proteins, establishing cytosolic acetyl-CoA as a metabolic rheostat that integrates nutrient signals with autophagic regulation. Given this established framework, the findings of the current study should be interpreted in the context of these earlier reports, and a discussion of these seminal studies should be included in the Introduction to strengthen the conceptual foundation.
2. Subcellular acetyl-CoA pools are heterogeneous, cytosolic acetyl-CoA playing major role in autophagy. In addition, histone acetylation in the nucleus also regulate acetylation. Therefore it is important to differentiate these different pools of acetyl-CoA for acetylation in response to impaired autophagy.
3. The authors investigated several key enzymes involved in acetyl-CoA biosynthesis, including ACLY, ACSS1/2, malonyl-CoA decarboxylase (MCD), and PDH, to elucidate the mechanisms underlying changes in hepatic acetyl-CoA levels. ACLY and ACSS2 primarily generate acetyl-CoA in the cytosol, whereas PDH and ACSS1 are mitochondrial enzymes contributing to mitochondrial acetyl-CoA pools (PMID: 40368140). MCD, which localizes to multiple cellular compartments, influences cytosolic acetyl-CoA availability by regulating malonyl-CoA levels, thereby affecting fatty acid synthesis. To strengthen the mechanistic clarity, the authors should explicitly distinguish the compartment-specific roles of these enzymes in acetyl-CoA production and discuss how NRF2-mediated transcriptional repression of these enzymes impacts acetyl-CoA levels in the cytosol versus mitochondria. This distinction is critical for understanding how NRF2 modulates protein acetylation and autophagy in a compartmentalized manner.
4. The mechanistic link between NRF2 activity and acetyl-CoA metabolism, as presented, remains insufficiently substantiated. While the authors propose that NRF2 represses the expression of key acetyl-CoA biosynthetic enzymes, direct evidence supporting this regulatory relationship is limited. To strengthen the proposed mechanism, additional experiments—such as chromatin immunoprecipitation (ChIP) to confirm NRF2 binding to the promoters of these genes, or NRF2 loss- and gain-of-function studies—are needed. Furthermore, given NRF2's central role in cellular redox homeostasis and stress responses, its connection to acetyl-CoA metabolism and autophagy should be clearly introduced and contextualized in the Introduction. Establishing this conceptual framework early on would enhance the mechanistic narrative and better support the study's conclusions.

Minor Comments:

- The reference list should be carefully verified, as several cited works could not be located or confirmed. Ensure all references are complete, accurate, and accessible.
- The descriptions of Figures 7B and 8C require correction. Specifically, KAT8—an acetyltransferase responsible for H4K16 acetylation and known to regulate autophagy—shows a significant reduction, which is not accurately reflected in the figure legends or main text.
- In Figure 6A, an increase in p62 levels is observed following 24 hours of fasting. This is unexpected and should be addressed, as p62 is typically degraded during autophagy. The authors should provide an explanation or discuss potential reasons for this finding.

Reviewer #2

(Remarks to the Author)

This manuscript proposes that autophagy regulates the hepatic acetylome by modulating intracellular acetyl-CoA levels, rather than through changes in acetyltransferase or deacetylase expression. The study identifies NRF2 as a key transcriptional repressor of acetyl-CoA biosynthetic enzymes in autophagy-deficient livers and demonstrates that acetyl-CoA supplementation restores acetylation and reduces liver injury.

These findings are novel and conceptually significant, with potential to influence thinking in liver pathophysiology, metabolic regulation, and therapeutic targeting of autophagy-related disorders. However, substantial revisions are necessary to address concerns regarding manuscript structure, data presentation, missing experiments, and reproducibility.

Major Comments

Manuscript Format and Clarity:

The manuscript reads like a thesis rather than a concise scientific article. It mixes background with results and includes excessive figure panels (12 main figures). Reorganization and figure consolidation are strongly recommended.

Missing or Incomplete Data:

Immunofluorescence validation of liver-specific Atg7 deletion is mentioned (Supplementary Figure 1A) but not shown. NRF2 activation is inferred from a single target gene (Nqo1), rather than direct assessment (e.g., nuclear localization or ChIP).

Figure 1E presents three WB lanes, but the quantification shows four dots—this inconsistency must be resolved.

Figure 3B states that liver histology was assessed, but no representative histological images are shown.

Only some western blots are quantified; this should be standardized across all figures or justified.

Mechanistic Gaps:

The authors propose that reduced acetylation contributes to liver injury, but only pan-acetylation is shown. No proteomic analysis is provided to identify specific hypoacetylated proteins.

The increase in p62 following fasting (Figure 6) contradicts the notion of autophagy activation and requires explanation.

Minor Comments

The Supplementary Information includes a figure labeled only “Thank you” (last page of Supplementary Figure 8). This is inappropriate and must be removed.

Provide clearer, more detailed figure legends, especially regarding sample size (n), type of replicates, and exact quantification methods.

In Supplementary Figure 1A, the immunoblot shows Nqo1 rather than NRF2; the text should clarify this.

Statistical and Reproducibility Concerns

Sample sizes and biological vs. technical replicates are not clearly reported across figures.

Quantitative data are missing for several western blot panels, reducing reproducibility.

The acetyl-CoA quantification methodology is not described in detail—include the specific assay type, normalization, and standard curve calibration.

Overall Recommendation

Major Revision

The study provides important mechanistic insights and is highly relevant to the field, but it requires substantial restructuring and completion of key data to meet publication standards.

Reviewer #3

(Remarks to the Author)

Summary

The manuscript “Autophagy Regulates the Hepatic Acetylome Through Modulating Acetyl-CoA Metabolite” by Baral et. al. investigates whether deficient autophagy in the liver is linked to any class of post-translational modifications (PTMs) that may be regulatory in the process. The authors find a reproducible decrease in protein acetylation in multiple models of autophagy deficiency, which they suggest is due to decreased acetyl-CoA levels from NRF2-mediated repression of acetyl-CoA metabolic genes. Restoration of acetyl-CoA rescues the hypoacetylation phenotype and mitigates liver injury.

Correlation of increased liver acetylation with processes that activate autophagy, such as fasting, lead the authors to suggest that acetylation is mechanistically involved with the autophagy process. While the work is very nice, it is a bit concerning that the authors overstate the novelty of their work and fail to cite an entire body of literature on autophagy and acetylation, both in the liver and in other tissues. Second, the authors would do well to more thoroughly incorporate previously published liver acetylproteomics datasets, from relevant conditions. I believe that this work would be of wide interest to the readers of Communications Biology and suggest that it be reconsidered for publication after addressing these and several additional minor concerns highlighted below.

Major Concerns

1) Novelty and citation: The introduction fails to reference a vast literature on the role of acetylation in autophagy. The authors should carefully examine these review articles (PMID:35435793; PMID:34237149; PMID:35042977; PMID:23466676) and incorporate the prior knowledge about the role of acetylation in regulating autophagy into the manuscript. Furthermore, there is a critical failure to cite several research articles (PMID:36753789; PMID:32473093; PMID:28558013) establishing a role for acetylation in regulating autophagy in the liver specifically. Previous work (PMID:32473093) has shown that acetyl-CoA derived from hepatic fatty acid oxidation inhibits autophagy, but it is not referenced. In explaining their rationale for investigating a number of PTMs in autophagy deficiency on lines 250-252 and 565-566, the authors should consider referencing reviews on what is previously known about different PTMs in autophagy (PMID:25365205; PMID: 25484070). On lines 360-362, previous work (PMID:26358839; PMID:23864654) demonstrating increased liver protein acetylation in fasting should be cited. Considering this vast literature, the authors should dampen their statement of novelty on lines 101-104.

2) Rescue of Acetyl-CoA levels: The line of work rescuing Acetyl-CoA levels in the liver is an interesting concept, but the approach seems flawed. Acetyl-CoA is a large, charged molecule that cannot cross cell membranes—including the plasma membrane of hepatocytes. This means that injected acetyl-CoA would not enter liver cells, but it would likely be degraded or excreted without significantly altering intracellular acetyl-CoA levels. There are no known transporters that shuttle acetyl-CoA across plasma membranes. All acetyl-CoA used in cells is produced intracellularly, primarily from pyruvate, fatty acid β -oxidation, and amino acid catabolism. To boost hepatic acetyl-CoA, the authors should administer the animals with precursors that promote endogenous production, such as acetate, ketogenic amino acids, fatty acids, pyruvate, or lactate. In addition to mouse work, the authors should investigate whether promoting acetyl-CoA production in the cultured AML12 hepatocytes abrogates the hypoacetylation induced by chloroquine and leupeptin. Previous work (PMID:2780628) has shown that medium chain fatty acids (eg. octanoate) are very effective at boosting acetyl-CoA (and acetylation) in AML12s.

Minor Concerns

1) The abstract seems too long. It is roughly 60% as long as the introduction and repeats a lot of the same information. The abstract should be made more succinct.

2) One line 250-252, it should be specified that the western blot screen used “pan” antibodies for each PTM type and are not sequence-specific.

3) More could be done to include data from acetyl-proteomics datasets that investigate conditions with altered autophagy, such as fasting (PMID:26358839; PMID:23864654), caloric restriction (PMID:23201123), and aging (PMID:35931320).

4) On lines 393-395, what statistical threshold was used for assessing significance?

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

This manuscript addresses an important topic by linking autophagy to global regulation of the hepatic acetylation via metabolic control of acetyl-CoA availability. The authors present compelling evidence that autophagy deficiency reduces protein acetylation and identify NRF2-dependent suppression of acetyl-CoA-producing enzymes as a central mechanism, with translational relevance to chronic liver disease. However, several key mechanistic gaps limit the depth of these conclusions. Notably, the functional interplay between NRF2 activation and autophagy is not directly examined; acetylation is largely assessed at a global level, with limited protein- and subcellular-resolution (aside from H3), and without evaluation of compartment-specific acetyl-CoA metabolism; and NAD⁺ metabolism is not explored despite uniform alterations in sirtuin expression. Addressing these issues would substantially strengthen the mechanistic rigor and physiological interpretation of the study. Detailed comments are listed below:

1. Protein acetylation and metabolism are linked (PMID: 40782900) and acetylation depends on multiple factors including fasting-fed states, circadian rhythms, type of diet. Therefore, it is critical to state in each experiment if animals had access to food and what time of the day the experiment was terminated and liver was collected.

2. Exogenous acetyl-CoA cannot increase intracellular acetyl-CoA levels because it is not cell-permeable and does not cross the plasma membrane. Instead, cellular acetyl-CoA pools are maintained by intracellular metabolic pathways (e.g., citrate-ACLY, acetate-ACSS2, pyruvate-PDH), rather than by direct acetyl-CoA uptake. Moreover, extracellular acetyl-CoA

is rapidly hydrolyzed to free acetate, further limiting the physiological relevance of acetyl-CoA injection as a strategy to modulate protein acetylation (Fig 8C and Fig 11).

3. The manuscript relies heavily on p62/SQSTM1 as a marker of autophagy, despite its well-recognized limitations as an indirect and context-dependent readout influenced by transcriptional regulation, proteasomal degradation, and stress signaling (e.g., NRF2). Consequently, p62 changes alone cannot distinguish between altered autophagy induction and defective autophagic flux. Although LC3 lipidation provides a more direct measure of autophagosome formation, it was assessed only in human samples, where LC3-I and LC3-II were not clearly resolved. Robust evaluation of autophagy will require properly resolved LC3-I/II immunoblots under conditions that assess autophagic flux.

4. The relationship between acetyl-CoA availability and protein acetylation is an important and well-recognized question; however, addressing this issue rigorously requires subcellular resolution of both acetyl-CoA pools and acetylation events (PMID: 31767181; PMID: 37224468; PMID: 40368140). The authors should more thoroughly review the existing literature and place their findings in the context of what is already known regarding the impact of diet on hepatic histone and non-histone protein acetylation patterns. Key prior studies in this area (PMID: 28077572; PMID: 33340539) are notably absent from the discussion and should be incorporated.

5. The term acetylome is conventionally used to describe proteome-wide acetylation analyses. As no proteomic data are presented in this study, use of this term is not appropriate. The authors should avoid acetylome and instead refer more precisely to protein acetylation or acetylation levels, as applicable.

6. Autophagy is primarily regulated by cytosolic acetyl-CoA levels through acetylation of key autophagy-related proteins in the cytosol. However, the present study does not provide data on acetyl-CoA levels or on the acetylation status of cytosolic proteins, limiting the strength of the conclusions regarding autophagy and Acetyl-CoA metabolism regulation.

7. H4K16 acetylation is known to regulate autophagy and metabolism at the transcriptional level. However, while the authors present data on H3 acetylation, they refer to H4 acetylation without providing corresponding experimental data, creating an inconsistency. The connection between H4K16 acetylation, metabolism, and autophagy should be addressed.

8. Protein acetylation is linked to cellular metabolism not only through its dependence on acetyl-CoA availability, but also through sirtuin-mediated deacetylation, which requires NAD⁺. The data in Figure 7E show reduced mRNA levels of all sirtuins in autophagy-deficient livers. Therefore, interpretations of acetylation-dependent regulation should also consider the potential contribution of altered NAD⁺ availability and sirtuin activity to changes in global acetylation. At a minimum, markers of cytosolic and mitochondrial redox state should be provided to assess subcellular NAD⁺/NADH ratios.

9. The section relating reduced hepatic acetyl-CoA levels to biosynthetic enzymes should consider the metabolic state and source of acetyl-CoA in each condition for each subcellular compartment. For instance, glucose is the main source of acetyl-CoA for histone acetylation in all conditions with some fatty acid contribution in fasted state. This needs to be specified in each experiment.

10. Recent work from the Wellen group has demonstrated that carnitine acetyltransferase (CrAT) can facilitate histone acetylation under certain conditions. Other studies have shown that when mitochondrial acetyl-CoA levels are elevated, flux through the CrAT pathway becomes substantial. Therefore, the authors should consider measuring CrAT expression and/or activity and evaluating its potential contribution to autophagy-dependent regulation of protein acetylation.

11. The authors' main conclusion is that NRF2 transcriptionally suppresses acetyl-CoA biosynthesis. However, the mechanistic link between impaired autophagy and NRF2 activation is not clearly established. As a result, the abstract figure does not accurately or clearly summarize the proposed model

Reviewer #2

(Remarks to the Author)

I recommend the publication of this manuscript. The authors have addressed all the reviewers' comments thoroughly, satisfactorily resolved all concerns, and the paper has significantly improved as a result

Reviewer #3

(Remarks to the Author)

Summary

The manuscript is much improved, and I recommend that it be accepted for publication in *Communications Biology* after successfully revising the text to address the following remaining concerns:

Major Concerns

1) Discussion of how autophagy impacts lysine acetylation:

- While proper incorporation of the literature is much improved, this very recent (hence easily missed) review highlights the bidirectional regulation between protein acetylation and autophagy (PMID: 41213956) and should be cited.

- I suggest restructuring the sentence starting on line 382 to read something like “diminished hepatic acetylation is **unlikely to be due** to altered acetyltransferase or deacetylation expression”, as this can't be known definitively and there are some expression changes noted. However, I would also add that “The uniformity of acetylation responses across subcellular compartments (Figure 2A) is consistent with this interpretation, as the mechanisms governing acetylation/deacetylation are highly compartment specific, whereas acetyl-CoA serves as a universal substrate for all known enzymatic and non-enzymatic reactions.

- The graphical abstract contains a critical chemical error. During lysine acetylation, acetyl-CoA donates only the acetyl group, with the CoA moiety (including the thiol) serving as the leaving group. Consequently, the PTM consists solely of an acetyl group linked to the lysine via the carbonyl carbon. See Figure 4 in this review (PMID: 26822488) for accurate depictions of lysine acetylation and acylation by other acyl-CoAs. In addition, the 'R' group shown on the 'metabolite' implies that acetyl-CoA is conjugated to another moiety. Because free acetyl-CoA is the substrate for all known enzymatic and non-enzymatic lysine acetylation reactions, it would be more accurate to depict acetyl-CoA without an attached R group.

2) Speculating on the possible effects of altered acetylation:

- The KEGG pathway enrichment analysis referenced in the sentence starting on line 594 should be included as a supplementary figure/table (with accompanying methods) and not simply listed as unpublished. It would be helpful to know what other pathways scored highly in this analysis in addition to the mitochondrial BCAA degradation pathway.

- It would help to note that while autophagy impacts protein acetylation patterns uniformly across subcellular compartments, the stoichiometry of these modifications differs substantially across organelles. As discussed in detail in this review (PMID: 39606008), well-established regulatory mechanisms exist for high-stoichiometry acetylation events in the nucleus, whereas the functional significance of the typically low-stoichiometry acetylation observed in mitochondria remains an active area of investigation.

- In fitting with the point above, the sentence starting on line 596 should be changed to something like "...impaired acetylation of mitochondrial enzymes in this pathway..."

- Similarly, the sentence starting on line 598 should be changed to something like "...identifying **the subcellular compartments and** the specific acetylated proteins..."

Minor Concerns

3) Even though it is stated later in the *Results* section, a brief explanation of why P62 was included in the immunoblots should be added when describing Figure 1 (perhaps moving the later text on this antibody).

4) line 272, it should be specified that the analysis is for "tyrosine" phosphorylation, as serine and threonine phosphorylation are prevalent PTMs.

5) On line 272, it should be stated that the analysis used two commercial antibodies "recognizing acetyl-lysine", as both N-terminal acetylation and cysteine acetylation also exist.

Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors have addressed several of the initial concerns; however, a key mechanistic issue remains unresolved regarding the interpretation of the acetyl-CoA experiments. The proposed explanation relies on indirect hydrolysis to acetate, yet the rationale for using acetyl-CoA instead of acetate is unclear. As currently presented, this experiment does not provide clear mechanistic insight. I therefore recommend minor revision to address this point, including appropriate controls to substantiate the proposed mechanism.

Reviewer #3

(Remarks to the Author)

The authors have satisfactorily addressed all my concerns. I recommend the manuscript be accepted for publication.

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Point by Point Response

Manuscript Title "Autophagy Regulates the Hepatic Acetylome Through Modulating Acetyl-CoA Metabolite"

Baral K et al 2025

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

This study provides novel insights into the intersection between autophagy and post-translational modifications (PTMs) in the liver, with a particular focus on protein acetylation. While the role of acetylation in autophagy is well recognized, the reverse impact of autophagy on acetylation remains underexplored. The authors employ multiple models of autophagy modulation to demonstrate that impaired autophagy leads to global changes in PTMs, notably a marked reduction in protein acetylation linked to suppressed acetyl-CoA biosynthesis. They further identify NRF2 as a transcriptional repressor of key enzymes in the acetyl-CoA production pathway, positioning it as a central mediator of this metabolic regulation. These findings highlight a previously unappreciated role of autophagy in maintaining hepatic acetylation status through metabolic control, with significant implications for liver disease pathogenesis and therapy.

Response 1: *Thank you for your thoughtful and encouraging remarks. We appreciate your recognition of the novelty and significance of our work.*

However, there are several concerns that need to be addressed:

1. The relationship between acetylation and autophagy is well established, i. e., when acetylation decreased autophagy increased (**PMID: 37224468**).

Previous studies also have shown that reduced cytosolic protein acetylation can activate autophagy, positioning acetylation as a nutrient-sensitive regulatory mechanism. Specifically, autophagy is inversely correlated with cytosolic acetyl-CoA levels, suggesting that fluctuations in acetyl-CoA availability modulate autophagic activity in response to cellular metabolic status (**Eisenberg et al., 2014**).

Under nutrient-rich conditions, cytosolic accumulation of acetyl-CoA enhances protein acetylation, which in turn suppresses autophagy, ultimately contributing to impaired metabolic homeostasis (**Schroeder et al., 2014**). This suppression is mediated through acetylation of key autophagy-related proteins, establishing cytosolic acetyl-CoA as a metabolic rheostat that integrates nutrient signals with autophagic regulation.

Given this established framework, the findings of the current study should be interpreted in the context of these earlier reports, and a discussion of these seminal studies should be included in the Introduction to strengthen the conceptual foundation.

Response 2: Thank you for highlighting this important point. We agree that the inverse relationship between cytosolic acetylation and autophagy is well established and appreciate the references provided (PMID: 37224468; Eisenberg et al., 2014; Schroeder et al., 2014). To address this, we have revised the Introduction to include a discussion of these seminal studies, emphasizing the role of cytosolic acetyl-CoA as a metabolic rheostat that integrates nutrient status with autophagic regulation.

Additionally, we have expanded the Discussion to interpret our findings within this conceptual framework, clarifying that while previous work focused on acetylation as a regulator of autophagy, our study reveals the reciprocal influence—how autophagy modulates acetylation through metabolic control. These modifications strengthen the theoretical basis and contextualize the novelty of our work.

2. Subcellular acetyl-CoA pools are heterogeneous, cytosolic acetyl-CoA playing major role in autophagy. In addition, histone acetylation in the nucleus also regulate acetylation. Therefore it is important to differentiate these different pools of acetyl-CoA for acetylation in response to impaired autophagy.

Response 3: Thank you for raising this important point. We agree that distinguishing subcellular acetyl-CoA pools is essential for interpreting acetylation changes. We have presented data on acetylome levels in subcellular fractions into the main **Figure 2A**. The analysis demonstrates that acetylation downregulation is not compartment-specific, as reductions were observed across multiple cellular compartments. This supports our conclusion that impaired autophagy induces global alterations in the acetylome rather than localized effects.

3. The authors investigated several key enzymes involved in acetyl-CoA biosynthesis, including ACLY, ACSS1/2, malonyl-CoA decarboxylase (MCD), and PDH, to elucidate the mechanisms underlying changes in hepatic acetyl-CoA levels. ACLY and ACSS2 primarily generate acetyl-CoA in the cytosol, whereas PDH and ACSS1 are mitochondrial enzymes contributing to mitochondrial acetyl-CoA pools (PMID: 40368140). MCD, which localizes to multiple cellular compartments, influences cytosolic acetyl-CoA availability by regulating malonyl-CoA levels, thereby affecting fatty acid synthesis. To strengthen the mechanistic clarity, the authors should explicitly distinguish the compartment-specific roles of these enzymes in acetyl-CoA production and discuss how NRF2-mediated transcriptional repression of these enzymes impacts acetyl-CoA levels in the cytosol versus mitochondria. This distinction is critical for understanding how NRF2 modulates protein acetylation and autophagy in a compartmentalized manner.

Response 4: Thank you for raising this important point. We agree that differentiating subcellular acetyl-CoA pools is critical for interpreting acetylation changes. We now include data examining acetylome levels in subcellular fractions in **Figure 2A**. The analysis clearly demonstrates that acetylation changes are not compartment-specific, as no significant differences were observed across distinct cellular compartments.

This supports the conclusion that impaired autophagy induces systemic alterations in the acetylome rather than localized effects. We have also incorporated these specific details into the Results and Discussion sections, highlighted in red text for clarity. Also, the relevant discussion of NRF2-mediated transcriptional repression of these enzymes and their impacts on acetyl-CoA levels has been provided in discussion section.

4. The mechanistic link between NRF2 activity and acetyl-CoA metabolism, as presented, remains insufficiently substantiated. While the authors propose that NRF2 represses the expression of key acetyl-CoA biosynthetic enzymes, direct evidence supporting this regulatory relationship is limited. To strengthen the proposed mechanism, additional experiments—such as chromatin immunoprecipitation (ChIP) to confirm NRF2 binding to the promoters of these genes, **or** NRF2 loss- and gain-of-function studies—are needed.

Response 5: *Thank you for this valuable suggestion. To strengthen the mechanistic link, we have now performed additional experiments as recommended. First, we identified potential NRF2 binding sites in the promoters of key acetyl-CoA biosynthetic enzymes (see revised **Figure 10C-10D**). We then conducted **ChIP assays**, which confirmed NRF2 binding to these promoter regions (see revised **Figure 10E**). Furthermore, genetic NRF2 deletion (loss-of-function) in the context of autophagy deficiency (**hepAtg7/NRF2^{-/-}**) reversed the ChIP DNA pull-down effect (see revised **Figure 10E**) and restored the expression of acetyl-CoA biosynthetic enzymes, as well as overall hepatic acetylome levels (see revised **Figure 10A-10B**). These results provide direct evidence supporting NRF2's role as a transcriptional repressor in this pathway.*

Furthermore, given NRF2's central role in cellular redox homeostasis and stress responses, its connection to acetyl-CoA metabolism and autophagy should be clearly introduced and contextualized in the Introduction. Establishing this conceptual framework early on would enhance the mechanistic narrative and better support the study's conclusions.

Response 6: *We have revised the **Introduction** to include NRF2's well-established antioxidative and stress-response functions, thereby providing a stronger conceptual framework for its connection to acetyl-CoA metabolism and autophagy. This contextualization enhances the mechanistic narrative and supports the significance of our findings.*

Minor Comments:

5) The reference list should be carefully verified, as several cited works could not be located or confirmed. Ensure all references are complete, accurate, and accessible.

Response 7: *Thank you for pointing this out. We have carefully reviewed and verified all references in the manuscript to ensure they are complete, accurate, and accessible. Any missing or incorrect citations have been corrected in the revised version.*

6) The descriptions of Figures 7B and 8C require correction. Specifically, KAT8—an acetyltransferase responsible for H4K16 acetylation and known to regulate autophagy—shows a significant reduction, which is not accurately reflected in the figure legends or main text.

Response 8: *Thank you for noting this discrepancy. We have corrected the descriptions of Figures 7B and 8C (revised Figure 7E) in both the figure legends and the main text to accurately reflect the significant reduction in KAT8, an acetyltransferase responsible for H4K16 acetylation and known to regulate autophagy. These revisions ensure consistency between the data presented and the accompanying descriptions.*

7) In Figure 6A, an increase in P62 levels is observed following 24 hours of fasting. This is unexpected and should be addressed, as P62 is typically degraded during autophagy. The authors should provide an explanation or discuss potential reasons for this finding.

Response 9: *Thank you for noting this discrepancy. We have re-examined P62 expressions and replaced the previous blot with a higher-quality image. The revised data now clearly shows that autophagy activation during starvation reduces P62 levels, consistent with its role as a bona fide autophagic substrate. Please see the updated Figure 6B, 6C in the revised manuscript.*

Reviewer #2 (Remarks to the Author):

This manuscript proposes that autophagy regulates the hepatic acetylome by modulating intracellular acetyl-CoA levels, rather than through changes in acetyltransferase or deacetylase expression. The study identifies NRF2 as a key transcriptional repressor of acetyl-CoA biosynthetic enzymes in autophagy-deficient livers and demonstrates that acetyl-CoA supplementation restores acetylation and reduces liver injury. These findings are novel and conceptually significant, with potential to influence thinking in liver pathophysiology, metabolic regulation, and therapeutic targeting of autophagy-related disorders.

Response 1: *Thank you for your positive and encouraging remarks. We appreciate your recognition of the novelty and conceptual significance of our work. This study addresses a critical gap by demonstrating that autophagy regulates the hepatic acetylome primarily through modulation of intracellular acetyl-CoA levels, rather than by altering acetyltransferase or deacetylase expression.*

However, substantial revisions are necessary to address concerns regarding manuscript structure, data presentation, missing experiments, and reproducibility.

Major Comments

1) Manuscript Format and Clarity: The manuscript reads like a thesis rather than a concise scientific article. It mixes background with results and includes excessive figure panels (12 main figures). Reorganization and figure consolidation are strongly recommended.

Response 2: *Thank you for this important feedback. We have thoroughly revised the manuscript to improve clarity and conciseness. Specifically, we have reorganized the **Introduction**, **Results**, and **Discussion** sections to clearly separate background information from experimental findings. The Results section has been rewritten to focus exclusively on the data and key observations. Additionally, we have consolidated*

*figures wherever possible, including merging (original) **Figures 7 and 8**, to reduce redundancies and streamline presentation. These changes ensure the manuscript reads as a concise scientific article rather than a thesis.*

2) Missing or Incomplete Data: Immunofluorescence validation of liver-specific Atg7 deletion is mentioned (Supplementary Figure 1A) but not shown.

Response 3: *Thank you for pointing this out. We have now included the missing immunostaining data for liver Atg7 and P62 protein as validation. These images are provided in the revised manuscript as **Supplementary Figure 1C**, confirming successful Atg7 knockout and autophagy impairment in the liver.*

3) NRF2 activation is inferred from a single target gene (NQO1), rather than direct assessment (e.g., nuclear localization or ChIP).

Response 4: *Thank you for this important comment. We have addressed this concern by providing additional data to directly assess NRF2 activation. Specifically, we now include Western blot analysis for NRF2 protein levels to complement the NQO1 data (new **Figure 10B**). Furthermore, we performed **ChIP assays** confirming NRF2 binding to the promoter region of **NQO1**, an established bona fide NRF2 target gene (new **Figure 10D**). These additions strengthen the evidence for NRF2 activation and its functional relevance in our study.*

4) Figure 1E presents three WB lanes, but the quantification shows four dots—this inconsistency must be resolved.

Response 5: *Thank you for pointing out this inconsistency. We have corrected **Figure 1E** by aligning the quantification with the Western blot data. Specifically, we now present three data points in the bar graph corresponding to the three lanes shown in the blot. This revision ensures consistency between the figure and its quantification.*

5) Figure 3B states that liver histology was assessed, but no representative histological images are shown.

Response 6: *Thank you for noting this omission. We have now included representative **H&E-stained liver histology images** for the wild-type group in **Figure 3B**. As shown, CQ treatment does not induce any pathological changes, confirming the absence of histological abnormalities. These additions provide visual support for the histology assessment described in the text.*

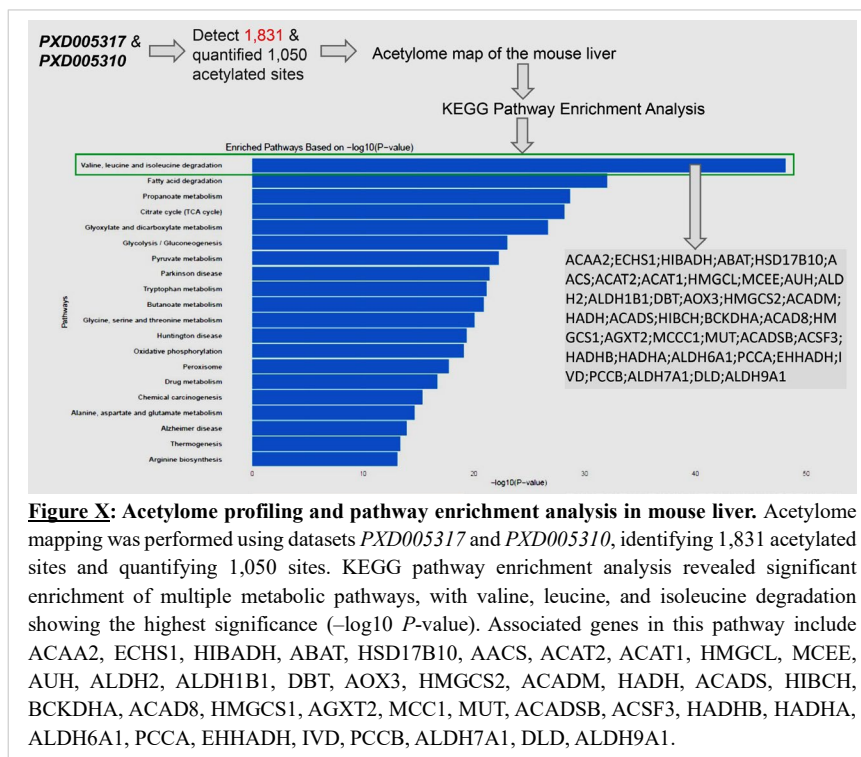
6) Only some western blots are quantified; this should be standardized across all figures or justified.

Response 7: *Thank you for highlighting this concern. We have now provided quantifications for Western blots wherever possible and necessary to substantiate the findings. Specifically, quantification has been added for **Figures 6, 8D, 8G, 8H** in addition to **Figures 1, 3, 4 5, and 12** as well as other relevant figures. In cases where quantification was not previously feasible, we repeated the Western blot experiments to ensure reliable data presentation. These revisions standardize the reporting and strengthen the robustness of our results.*

7) Mechanistic Gaps: The authors propose that reduced acetylation contributes to liver injury, but only pan-acetylation is shown. No proteomic analysis is provided to identify specific hypoacetylated proteins.

Response 8: *Thank you for this insightful comment. While a full proteomic analysis of hypoacetylated proteins is beyond the scope of the current study, we agree that this represents an important future direction. To address this, we have added statements in the **Discussion** and **Results** sections outlining these plans.*

*Specifically, we performed preliminary proteomic analysis of the acetylated hepatic proteome using datasets **PXD005317** and **PXD005310**, which revealed that enzymes involved in the branched-chain amino acid (BCAA) pathway were among the most highly acetylated (see preliminary data provided for reviewer reference only) (**Figure X**). Considering the overall downregulation of hepatic proteome in autophagy-deficient liver, we anticipate that these BCAA pathway enzymes could be significantly affected and may play a critical role in liver pathophysiology. Ongoing studies aim to validate these findings and explore their functional implications.*



8) The increase in p62 following fasting (Figure 6) contradicts the notion of autophagy activation and requires explanation.

Response 9: *Thank you for noting this discrepancy. We have re-examined p62 expression and replaced the previous blot with a higher-quality image. The revised data now clearly shows that autophagy activation during starvation reduces p62 levels, consistent with its role as a bona fide autophagic substrate. Please see the updated **Figure 6A** and **Figure 6B** in the revised manuscript.*

Minor Comments

9) The Supplementary Information includes a figure labeled only “Thank you” (last page of Supplementary Figure 8). This is inappropriate and must be removed.

Response 10: *Thank you for pointing this out. We have removed the slide labeled “Thank you” from the Supplementary Information to ensure that all supplementary figures are appropriate and relevant to the manuscript.*

10) Provide clearer, more detailed figure legends, especially regarding sample size (n), type of replicates, and exact quantification methods.

Response 11: *Thank you for this suggestion. We have revised all figure legends to provide clearer and more detailed information, including sample size (n), type of replicates (biological vs. technical), and the exact quantification methods used (e.g., densitometry for Western blots, normalization procedures, and statistical tests). These modifications ensure transparency and reproducibility of the data presented.*

11) In Supplementary Figure 1A, the immunoblot shows Nqo1 rather than NRF2; the text should clarify this.

Response 12: *Thank you for pointing this out. We have revised the text (line 227-229) to clarify that **Supplementary Figure 1A** shows the immunoblot for **NQO1**, a downstream NRF2 target, rather than NRF2 itself. This correction ensures consistency between the figure and the description.*

12) Statistical and Reproducibility Concerns-Sample sizes and biological vs. technical replicates are not clearly reported across figures. Quantitative data are missing for several western blot panels, reducing reproducibility. The acetyl-CoA quantification methodology is not described in detail—include the specific assay type, normalization, and standard curve calibration.

Response 13: *Thank you for highlighting these important points. We have addressed them as follows: 1) **Sample Sizes and Replicates:** We have now clearly indicated sample sizes and distinguished biological versus technical replicates in all figure legends and the Methods section for transparency and reproducibility. 2) **Western Blot Quantification:** Quantitative data have been added for all Western blot panels wherever possible, and figure legends have been updated accordingly. 3) **Acetyl-CoA Quantification Methodology:** We have expanded the Methods section to include detailed information on the assay type, normalization strategy, and standard curve calibration used for acetyl-CoA measurement. These additions ensure clarity and reproducibility of the experimental approach.*

Overall Recommendation-Major Revision: The study provides *important mechanistic insights and is highly relevant to the field*, but it requires substantial restructuring and completion of key data to meet publication standards.

Reviewer #3 (Remarks to the Author):

Summary-The manuscript “Autophagy Regulates the Hepatic Acetylome Through Modulating Acetyl-CoA Metabolite” by Baral et. al. investigates whether deficient autophagy in the liver is linked to any class of post-translational modifications (PTMs) that may be regulatory in the process. The authors find a reproducible decrease in protein acetylation in multiple models of autophagy deficiency, which they suggest is due to decreased acetyl-CoA levels from NRF2-mediated repression of acetyl-CoA metabolic genes. Restoration of acetyl-CoA rescues the hypoacetylation phenotype and mitigates liver injury. Correlation of

increased liver acetylation with processes that activate autophagy, such as fasting, lead the authors to suggest that acetylation is mechanistically involved with the autophagy process. While the work is very nice, it is a bit concerning that the authors overstate the novelty of their work and fail to cite an entire body of literature on autophagy and acetylation, both in the liver and in other tissues. Second, the authors would do well to more thoroughly incorporate previously published liver acetylproteomics datasets, from relevant conditions. I believe that this work would be of wide interest to the readers of Communications Biology and suggest that it be reconsidered for publication after addressing these and several additional minor concerns highlighted below.

Response 1: *Thank you for your thoughtful and encouraging evaluation of our work. We appreciate your recognition of the significance of our findings and your suggestions for improvement. We acknowledge the concern regarding overstating novelty and the omission of relevant literature. We have addressed the concerns by: 1) **Expanding the literature review** to include key studies on autophagy and acetylation in liver and other tissues, ensuring proper context. 2) **Integrating previously published liver acetyl proteomics datasets** into the Discussion to strengthen relevance and consistency. 3) **Clarifying novelty** by emphasizing that our main contribution is identifying NRF2-mediated repression of acetyl-CoA biosynthetic enzymes as a mechanism linking autophagy to global acetylation changes. These revisions provide a balanced interpretation and reinforce the mechanistic and translational significance of our findings.*

Major Concerns

1) Novelty and citation: The introduction fails to reference a vast literature on the role of acetylation in autophagy. The authors should carefully examine these review articles (PMID:35435793; PMID:34237149; PMID:35042977; PMID:23466676) and incorporate the prior knowledge about the role of acetylation in regulating autophagy into the manuscript.

Furthermore, there is a critical failure to cite several research articles (PMID:36753789; PMID:32473093; PMID:28558013) establishing a role for acetylation in regulating autophagy in the liver specifically. Previous work (PMID:32473093) has shown that acetyl-CoA derived from hepatic fatty acid oxidation inhibits autophagy, but it is not referenced.

In explaining their rationale for investigating a number of PTMs in autophagy deficiency on lines 250-252 and 565-566, the authors should consider referencing reviews on what is previously known about different PTMs in autophagy (PMID:25365205; PMID: 25484070).

On lines 360-362, previous work (PMID:26358839; PMID:23864654) demonstrating increased liver protein acetylation in fasting should be cited. Considering this vast literature, the authors should dampen their statement of novelty on lines 101-104.

Response 2: *We appreciate the reviewer's insightful comments and agree that incorporating prior literature will strengthen the manuscript. We have carefully reviewed the suggested articles and made the following changes:*

1. Added Citations:

- *We have included references to the comprehensive reviews on the role of acetylation in autophagy (PMID: 35435793; 34237149; 35042977; 23466676) in the Introduction section to provide appropriate context.*
- *We have cited key research articles (PMID: 36753789; 32473093; 28558013) that establish the role of acetylation in regulating autophagy in the liver. Specifically, we now reference previous work (PMID: 32473093) showing that acetyl-CoA derived from hepatic fatty acid oxidation inhibits autophagy.*
- *In explaining our rationale for investigating multiple PTMs (lines 250–252 and 565–566), we have added citations to reviews summarizing the role of different PTMs in autophagy (PMID: 25365205; 25484070).*
- *On lines 360–362, we have cited studies demonstrating increased liver protein acetylation during fasting (PMID: 26358839; 23864654).*

2. Textual Modification of Novelty Statement (Lines 101–104): *The original statement suggested a high degree of novelty without acknowledging prior work. We have revised this section to reflect that while previous studies have established a role for acetylation in autophagy regulation, our work provides additional mechanistic insights into how autophagy function in turn influences acetylome in the liver. The revised text reads:*

“Together, these results suggest an important role for autophagy in influencing the hepatic acetylome, potentially through metabolic regulation of acetyl-CoA. These findings add to the growing body of evidence on autophagy’s impact on liver metabolism and may have implications for understanding liver pathophysiology and identifying therapeutic strategies.”

2) Rescue of Acetyl-CoA levels: The line of work rescuing Acetyl-CoA levels in the liver is an interesting concept, but the approach seems flawed. Acetyl-CoA is a large, charged molecule that cannot cross cell membranes—including the plasma membrane of hepatocytes. This means that injected acetyl-CoA would not enter liver cells, but it would likely be degraded or excreted without significantly altering intracellular acetyl-CoA levels. There are *no known transporters that shuttle acetyl-CoA across plasma membranes*. All acetyl-CoA used in cells is produced intracellularly, primarily from pyruvate, fatty acid β -oxidation, and amino acid catabolism. To boost hepatic acetyl-CoA, the authors should administer the animals with precursors that promote endogenous production, such as acetate, ketogenic amino acids, fatty acids, pyruvate, or lactate. In addition to mouse work, the authors should investigate whether promoting acetyl-CoA production in the cultured AML12 hepatocytes abrogates the hypoacetylation induced by chloroquine and leupeptin. Previous work (PMID:2780628) has shown that medium chain fatty acids (eg. octanoate) are very effective at boosting acetyl-CoA (and acetylation) in AML12s.

Response 3: *We appreciate the reviewer’s insightful comments. To address this concern, we performed additional interventional experiments both **in vivo** and **in vitro**. In *Atg7^{HepErt2}* mice, **acetate supplementation** effectively rescued the hepatic acetylome (**Figure 8F, 8G**) and, importantly, alleviated liver injury and associated pathologies (**Figure 11D, 11E**), similar to the effect observed with acetyl-CoA intervention.*

Likewise, in AML12 hepatocytes treated with autophagy inhibitor (CQ), acetate supplementation produced comparable restoration of acetylation levels (**Figure 8H**). These findings reinforce the role of acetyl-CoA availability in maintaining protein acetylation and liver health.

Minor Concerns

3) The abstract seems too long. It is roughly 60% as long as the introduction and repeats a lot of the same information. The abstract should be made more succinct.

Response 4: *We appreciate the reviewer's feedback. We have revised the abstract to make it more succinct while retaining the essential findings and significance of the study.*

4) One line 250-252, it should be specified that the western blot screen used “pan” antibodies for each PTM type and are not sequence-specific.

Response 5: *We thank the reviewer for pointing this out. We have revised the text on lines 250–252 to specify that the western blot screen utilized “pan” antibodies for each PTM type, which are not sequence-specific.*

5) More could be done to include data from acetyl-proteomics datasets that investigate conditions with altered autophagy, such as fasting (**PMID:26358839**; **PMID:23864654**), caloric restriction (**PMID:23201123**), and aging (**PMID:35931320**).

Response 6: *We appreciate the reviewer for sharing this critical information. These datasets provide valuable insights and will be instrumental for future studies aimed at determining specific protein acetylation changes in autophagy-altered liver and correlating these modifications with liver physiology and pathologies associated with autophagy deficiency. Our current manuscript primarily focuses on global acetylation changes and their metabolic underpinnings, laying the foundation for more detailed proteomic analyses in future work. Such studies will help delineate the precise acetylation targets and pathways that contribute to liver dysfunction under autophagy-deficient conditions.*

6) On lines 393-395, what statistical threshold was used for assessing significance?

Response 7: *We thank the reviewer for this valuable suggestion. The heatmap has been updated to display standard error (SE) for each gene. Statistical significance for all comparisons was assessed using Student's t-test, with a threshold of $p < 0.05$. Significance is indicated as follows: n.s., not significant; * $P \geq 0.05$; ** $P \geq 0.01$; *** $P \geq 0.001$ (see revised Figure 7 legend).*

Point by Point Response

Manuscript Title "Autophagy Regulates the Hepatic Acetylome Through Modulating Acetyl-CoA Metabolite" COMMSBIO-25-5728A

Baral K et al 2026

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

This manuscript addresses an important by linking autophagy to global regulation of the hepatic acetylation via metabolic control of acetyl-CoA availability. The authors present compelling evidence that autophagy deficiency reduces protein acetylation and identify NRF2-dependent suppression of acetyl-CoA-producing enzymes as a central mechanism, with translational relevance to chronic liver disease.

Response 1: *We thank the reviewer for the positive evaluation of our work. We appreciate the recognition of our findings linking autophagy deficiency to reduced hepatic protein acetylation through NRF2-mediated suppression of acetyl-CoA-producing enzymes and their relevance to chronic liver disease.*

However, several key mechanistic gaps limit the depth of these conclusions. Notably, the functional interplay between NRF2 activation and autophagy is not directly examined; acetylation is largely assessed at a global level, with limited protein- and subcellular-resolution (aside from H3), and without evaluation of compartment-specific acetyl-CoA metabolism; and NAD⁺ metabolism is not explored despite uniform alterations in sirtuin expression. Addressing these issues would substantially strengthen the mechanistic rigor and physiological interpretation of the study.

Response 2: *We thank the reviewer for these insightful comments and fully agree that the points raised represent important avenues for deeper mechanistic investigation. Below, we provide detailed responses to each aspect of the critique.*

Detailed comments are listed below:

1. Protein acetylation and metabolism are linked (**PMID: 40782900**) and acetylation depends on multiple factors including fasting-fed states, circadian rhythms, type of diet. Therefore, it is critical to state in each experiment if animals had access to food and what time of the day the experiment was terminated and liver was collected.

Response 3: *We thank the reviewer for highlighting the importance of nutritional and circadian factors in interpreting hepatic acetylation. In the revised manuscript, we now specify the feeding status and exact tissue collection time for all in vivo experiments. Unless otherwise noted, mice were maintained on a*

controlled light–dark cycle with ad libitum access to food, and livers were collected consistently between 12:00 PM and 2:00 PM. These details are provided in the Materials and Methods section (lines 122–125).

2. Exogenous acetyl-CoA cannot increase intracellular acetyl-CoA levels because it is not cell-permeable and does not cross the plasma membrane. Instead, cellular acetyl-CoA pools are maintained by intracellular metabolic pathways (e.g., citrate–ACLY, acetate–ACSS2, pyruvate–PDH), rather than by direct acetyl-CoA uptake. Moreover, extracellular acetyl-CoA is rapidly hydrolyzed to free acetate, further limiting the physiological relevance of acetyl-CoA injection as a strategy to modulate protein acetylation (**Fig 8C** and **Fig 11**).

Response 4: *We thank the reviewer for this important clarification. We agree that **exogenous acetyl-CoA is not cell permeable** and therefore cannot directly increase intracellular acetyl-CoA levels. The most plausible mechanism underlying our observations is the **rapid extracellular hydrolysis of acetyl-CoA to free acetate**. This acetate can then be readily taken up by hepatocytes and converted back to acetyl-CoA via **ACSS2-dependent pathways**, providing a biologically credible explanation for the observed increase in protein acetylation.*

*Importantly, while **ACSS2 expression is reduced**, it is **not completely depleted** in our model (Fig. 9F), supporting the feasibility of this mechanism. We have revised the manuscript to clarify this point and to strengthen the interpretation of the in vivo acetyl-CoA injection experiments (see Discussion, lines 626–635).*

3. The manuscript relies heavily on p62/SQSTM1 as a marker of autophagy, despite its well-recognized limitations as an indirect and context-dependent readout influenced by transcriptional regulation, proteasomal degradation, and stress signaling (e.g., NRF2). Consequently, p62 changes alone cannot distinguish between altered autophagy induction and defective autophagic flux. Although LC3 lipidation provides a more direct measure of autophagosome formation, it was assessed only in human samples, where LC3-I and LC3-II were not clearly resolved. Robust evaluation of autophagy will require properly resolved LC3-I/II immunoblots under conditions that assess autophagic flux.

Response 5: *We appreciate the reviewer’s concern and **partially agree**. we respectfully emphasize that **p62 accumulation is a well-established and guideline-supported indicator of autophagy deficiency when interpreted in appropriate biological context**. The current **autophagy consensus guidelines** (Klionsky et al., 2021; PMID: 33634751) explicitly recognize p62 as a reliable marker of impaired autophagy, particularly when its accumulation is robust and consistent across models.*

*Consistent with these guidelines, multiple independent studies—including our own prior work—have demonstrated **robust p62 accumulation under conditions of defective autophagy**, supporting its utility as a biologically meaningful readout (PMIDs: 30520052, 29558368, 32382012, 36810855, 24815875).*

Importantly, in our study, p62 changes are observed in experimental settings where autophagy impairment is mechanistically expected, further strengthening their interpretation.

*To directly address the reviewer’s concern and to provide a more comprehensive assessment of autophagy, we have now **added LC3 immunoblot analyses** to the revised manuscript (Figure. 6). LC3-I to LC3-II conversion is a widely accepted marker of autophagosome formation, and the **combined analysis of LC3***

and p62 is recommended as best practice for evaluating autophagy status. These additions allow us to interpret p62 changes in conjunction with autophagosome formation rather than in isolation.

*Furthermore, we evaluated autophagy under **well-established autophagy-activating conditions**, including fasting/starvation and genetic perturbation of **Beclin-1 (Becn1)**. These experimental contexts are widely recognized to reproducibly induce autophagy and have been validated extensively in literature as well as in our prior studies (PMID: 30520052). In these settings, we observe **coordinated and concordant changes in LC3 and p62**, providing strong evidence that our readouts reflect genuine alterations in autophagy activity rather than secondary stress- or transcription-driven effects.*

*Together, the inclusion of LC3 analyses, the use of established autophagy-inducing paradigms, and the interpretation of p62 in a biologically appropriate context substantially **strengthen the rigor of our autophagy assessment**. These revisions ensure that our conclusions are consistent with current field standards and directly address the reviewer's concern regarding autophagic flux interpretation.*

4. The relationship between acetyl-CoA availability and protein acetylation is an important and well-recognized question; however, addressing this issue rigorously requires subcellular resolution of both acetyl-CoA pools and acetylation events (PMID: 31767181; PMID: 37224468; PMID: 40368140). The authors should more thoroughly review the existing literature and place their findings in the context of what is already known regarding the *impact of diet on hepatic histone and non-histone protein acetylation patterns*. Key prior studies in this area (PMID: 28077572; PMID: 33340539) are notably absent from the discussion and should be incorporated.

Response 6: *We thank the reviewer for highlighting the importance of **subcellular acetyl-CoA compartmentalization** and its role in regulating protein acetylation. We fully agree that a rigorous dissection of the relationship between acetyl-CoA availability and protein acetylation requires **compartment-specific analysis of acetyl-CoA pools and acetylation events**, as demonstrated in prior studies (PMIDs: 31767181, 37224468, 40368140). These works elegantly show how mitochondrial versus nucleocytosolic acetyl-CoA dynamics differentially influence histone and non-histone acetylation. The primary objective of our study, however, was to define the **autophagy-dependent regulation of hepatic acetyl-CoA biosynthetic enzymes** and its impact on **global protein acetylation**. Consistent with this goal, our data demonstrate that **autophagy deficiency leads to reduced total acetyl-CoA levels**, accompanied by a **broad decrease in protein acetylation across cytosolic, nuclear, and membrane fractions** (Figure 2A). Thus, while high-resolution subcellular metabolomic profiling would be informative, it would provide **incremental rather than transformative insight** relative to our central mechanistic conclusions. We also appreciate the reviewer's suggestion to better contextualize our findings within the literature on **diet-dependent regulation of hepatic histone and non-histone acetylation** (PMIDs: 28077572, 33340539). In the current study, all comparisons were performed between autophagy-intact and autophagy-deficient mice maintained on the **same standard diet**, and we did not investigate the effects of specific dietary interventions. Therefore, a detailed analysis of diet-driven acetylation patterns was beyond the scope of this work.*

*Nonetheless, we agree that this is an important area of investigation. Because dietary conditions such as **high-fat diet and chronic alcohol exposure are known to impair autophagy**, future studies will be essential*

*to determine whether **NRF2-mediated repression of acetyl-CoA biosynthetic enzymes** contributes to altered protein acetylation under these conditions. We have now incorporated this discussion and the relevant citations (PMIDs: 28077572, 33340539) into the revised manuscript (Discussion, lines 646–651), thereby strengthening the contextualization of our findings and clarifying how our work complements existing studies on acetyl-CoA metabolism and protein acetylation.*

5. The term acetylome is conventionally used to describe proteome-wide acetylation analyses. As no proteomic data are presented in this study, use of this term is not appropriate. The authors should avoid acetylome and instead refer more precisely to protein acetylation or acetylation levels, as applicable.

Response 7: *We thank the reviewer for this helpful clarification. We agree that “acetylome” conventionally refers to proteome-wide acetylation profiling derived from mass-spectrometry–based analyses. Because our study does not include proteomic acetylation datasets, we have revised the manuscript to avoid use of the term “acetylome” when describing our experimental findings. Throughout the revised text, we now refer more precisely to **protein acetylation**, **histone acetylation**, or **acetylation levels** as appropriate. The term “acetylome” is retained only when referring to the conceptual framework or previously published proteome-wide acetylation studies.*

6. Autophagy is primarily regulated by cytosolic acetyl-CoA levels through acetylation of key autophagy-related proteins in the cytosol. However, the present study does not provide data on acetyl-CoA levels or on the acetylation status of cytosolic proteins, limiting the strength of the conclusions regarding autophagy and Acetyl-CoA metabolism regulation.

Response 8: *We thank the reviewer for this insightful comment. We have examined cytosolic protein acetylation and incorporated these data into the manuscript (Figure. 2A). In addition, the immunofluorescence images in Figure. 2B demonstrate that acetylated proteins are markedly reduced not only in the cytosol but also in the nucleus and other subcellular compartments in autophagy-deficient liver. These results strengthen and complement our global acetylation analyses by providing direct evidence of reduced cytosolic acetylation under autophagy-deficient conditions. We agree that measuring acetyl-CoA levels directly would further clarify the mechanistic link, and we highlight this as a priority for future studies.*

7. H4K16 acetylation is known to regulate autophagy and metabolism at the transcriptional level. However, while the authors present data on H3 acetylation, they refer to H4 acetylation without providing corresponding experimental data, creating an inconsistency. The connection between H4K16 acetylation, metabolism, and autophagy should be addressed.

Response 9: *We thank the reviewer for raising this point. In the revised manuscript, we restrict our experimental analysis and conclusions to **H3 acetylation**, as these were the only histone acetylation marks directly measured in the study. We clarify that **we do not present H4 acetylation or H4K16ac data**. The mention of **H4K16 acetylation** appears only in the context of prior knowledge—specifically, to note its established association with **KAT8/MOF function and autophagy regulation**, as referenced in **Figure. 7B***

and the Results section (**line 403**). We have revised the text to ensure this distinction is clear and to avoid any unintended implication that H4K16ac was experimentally assessed in our study.

8. Protein acetylation is linked to cellular metabolism not only through its dependence on acetyl-CoA availability, but also through sirtuin-mediated deacetylation, which requires NAD^+ . The data in Figure 7E show reduced mRNA levels of all sirtuins in autophagy-deficient livers. Therefore, interpretations of acetylation-dependent regulation should also consider the potential contribution of altered NAD^+ availability and sirtuin activity to changes in global acetylation. At a minimum, *markers of cytosolic and mitochondrial redox state should be provided to assess subcellular $NAD^+/NADH$ ratios.*

Response 10: *We thank the reviewer for highlighting the important interplay between acetyl-CoA availability, NAD^+ metabolism, and sirtuin-mediated deacetylation in regulating protein acetylation. Sirtuins (SIRT1–SIRT7) are NAD^+ -dependent deacetylases that remove acetyl groups from lysine residues; therefore, reduced NAD^+ levels or decreased sirtuin expression/activity would be expected to diminish deacetylase capacity and potentially increase protein acetylation. As shown in Figure. 7E–7F, several sirtuins are downregulated in autophagy-deficient livers, indicating that enhanced deacetylation is unlikely to account for the observed reduction in protein acetylation.*

In the present study, we did not directly measure NAD^+ abundance, subcellular $NAD^+/NADH$ ratios, or sirtuin enzymatic activity, as these assessments require dedicated compartments-specific metabolomic approaches beyond the scope of the current work.

To address the reviewer's point, we have expanded the Discussion(line-592-609) to acknowledge that altered NAD^+ availability and sirtuin function may contribute to the acetylation landscape, representing a complementary regulatory layer alongside the acetyl-CoA-dependent mechanism described here. We also identify future studies evaluating cytosolic and mitochondrial $NAD^+/NADH$ ratios and direct sirtuin activity assays as important next steps toward defining how redox metabolism intersects with autophagy-dependent regulation of hepatic protein acetylation.

9. The section relating reduced hepatic acetyl-CoA levels to biosynthetic enzymes should consider the metabolic state and source of acetyl-CoA in each conditions for each subcellular compartment. For instance, glucose is the main source of acetyl-CoA for histone acetylation in all conditions with some fatty acid contribution in fasted state. This needs to be specified in each experiment.

Response 11: *We thank the reviewer for highlighting the importance of metabolic state and substrate availability in interpreting acetyl-CoA dynamics across subcellular compartments. We agree that glucose-derived acetyl-CoA is the predominant contributor to histone acetylation under fed conditions, with fatty acids playing a greater role during fasting. In the revised manuscript, we now specify both the nutritional state and the sampling time for all in vivo experiments and clarify the expected metabolic sources of acetyl-CoA under the conditions studied. These details have been added to the Materials and Methods (lines 124–125) and the Results section (lines 459–464).*

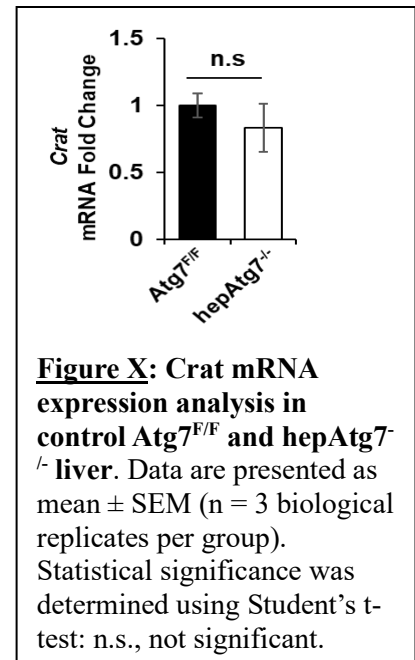
10. Recent work from the Wellen group has demonstrated that carnitine acetyltransferase (CrAT) can facilitate histone acetylation under certain conditions. Other studies have shown that when mitochondrial acetyl-CoA levels are elevated, flux through the CrAT pathway becomes substantial. Therefore, the authors should consider measuring CrAT expression and/or activity and evaluating its potential contribution to autophagy-dependent regulation of protein acetylation.

Response 12: *We appreciate the reviewer's suggestion regarding the potential role of carnitine acetyltransferase (CrAT) in regulating acetyl-CoA flux and protein acetylation. While CrAT has been shown to facilitate nucleo-cytosolic acetyl-CoA availability under conditions of elevated mitochondrial acetyl-CoA, our data do not support a major role for this pathway in our model.*

Specifically, Crat mRNA expression did not significantly changed in autophagy-deficient livers (Figure X; reviewer reference only).

Moreover, we observe an overall reduction in total hepatic acetyl-CoA, arguing against the mitochondrial acetyl-CoA excess that typically drives CrAT-mediated acetyl flux. Although direct assessment of CrAT protein levels or activity would be informative, such analyses are beyond the scope of this study.

Collectively, our data suggest that CrAT is unlikely to substantially contribute to the autophagy-dependent changes in protein acetylation reported here.



11. The authors' main conclusion is that NRF2 transcriptionally suppresses acetyl-CoA biosynthesis. However, the mechanistic link between impaired autophagy and NRF2 activation is not clearly established. As a result, the abstract figure does not accurately or clearly summarize the proposed model.

Response 13: *We thank the reviewer for raising this point. Although our study focuses on the downstream NRF2-dependent repression of acetyl-CoA-producing enzymes, the mechanistic link between autophagy deficiency and NRF2 activation is well established in the field. Prior studies from multiple groups—including Masaki Komatsu (PMID-20173742), Wen-Xing Ding (PMID-24815875), and our own (PMID-30520052;29558368;36810855;37507374)—have shown that loss of autophagy leads to p62/SQSTM1 accumulation, which competes with KEAP1 and results in NRF2 stabilization. Because this pathway is well characterized, we did not re-examine it experimentally here.*

To improve clarity, we now briefly describe this autophagy-p62-NRF2 mechanism in the Results section (lines 473-477). We have also revised the abstract figure to more accurately reflect the components of the model directly supported by our data.

Reviewer #2 (Remarks to the Author):

I recommend the publication of this manuscript. The authors have addressed all the reviewers' comments

thoroughly, satisfactorily resolved all concerns, and the paper has significantly improved as a result

Response 1: *We sincerely thank the reviewer for the positive evaluation of our revised manuscript. We appreciate your acknowledgment that we have thoroughly addressed the earlier comments and concerns. Your supportive remarks and recommendations for publication are greatly appreciated.*

Thank you for your time and constructive feedback, which helped us improve the quality and clarity of the paper.

Reviewer #3 (Remarks to the Author):

Summary

The manuscript is much improved, and I recommend that it be accepted for publication in *Communications Biology* after successfully revising the text to address the following remaining concerns:

Response 1: *We thank the reviewer for their positive assessment of the revised manuscript and for recommending it for publication. We appreciate the remaining comments and have carefully revised the text to address each point in detail. All modifications have been incorporated into the manuscript, and corresponding explanations are provided below. We believe these additional revisions have further improved the clarity and rigor of the work.*

Major Concerns

1) *Discussion of how autophagy impacts lysine acetylation:*

- While proper incorporation of the literature is much improved, this very recent (hence easily missed) review highlights the bidirectional regulation between protein acetylation and autophagy (PMID: **41213956**) and should be cited.

Response 2: *We thank the reviewer for highlighting this important and timely review discussing the bidirectional regulatory interplay between autophagy and protein acetylation. The manuscript has been updated accordingly, and the new citation (#57) has been incorporated into the revised text.*

- I suggest restructuring the sentence starting on **line 382** to read something like “*diminished hepatic acetylation is **unlikely to be due** to altered acetyltransferase or deacetylation expression*”, as this can’t be known definitively and there are some expression changes noted. However, I would also add that “*The uniformity of acetylation responses across subcellular compartments (Figure 2A) is consistent with this interpretation, as the mechanisms governing acetylation/deacetylation are highly compartment specific, whereas acetyl-CoA serves as a universal substrate for all known enzymatic and non-enzymatic reactions.*”

Response 3: We appreciate the reviewer's thoughtful suggestion and have revised the sentence accordingly (see line 411-415). The text has been updated to read: *"Diminished hepatic acetylation is unlikely to be due to altered acetyltransferase or deacetylase expression,"* which more accurately reflects that expression changes cannot be definitively ruled out. In addition, as recommended, we have added the statement: *"The uniformity of acetylation responses across subcellular compartments (Figure.2A) is consistent with this interpretation, as the mechanisms governing acetylation and deacetylation are highly compartment-specific, whereas acetyl-CoA serves as a universal substrate for all known enzymatic and non-enzymatic acetylation reactions."*

We agree that this addition strengthens the mechanistic reasoning and have incorporated these changes into the revised manuscript.

- The graphical abstract contains a critical chemical error. During lysine acetylation, acetyl-CoA donates only the acetyl group, with the CoA moiety (including the thiol) serving as the leaving group. Consequently, the PTM consists solely of an acetyl group linked to the lysine via the carbonyl carbon. See Figure 4 in this review (PMID: 26822488) for accurate depictions of lysine acetylation and acylation by other acyl-CoAs.

In addition, the 'R' group shown on the 'metabolite' implies that acetyl-CoA is conjugated to another moiety. Because free acetyl-CoA is the substrate for all known enzymatic and non-enzymatic lysine acetylation reactions, it would be more accurate to depict acetyl-CoA without an attached R group.

Response 4: We thank the reviewer for identifying the chemical inaccuracy in the graphical abstract. We agree that acetyl-CoA donates only the acetyl group during lysine acetylation, with CoA serving as the leaving group. The post-translational modification therefore consists solely of the acetyl moiety linked to the lysine ϵ -amine via the carbonyl carbon, as accurately depicted in Figure 4 of Baeza et al. (2016). We have revised the graphical abstract to correctly illustrate this chemistry.

Additionally, we appreciate the reviewer's point regarding the misleading "R" group on the metabolite depiction. Because free acetyl-CoA is the universal substrate for all known enzymatic and non-enzymatic lysine acetylation reactions, the inclusion of an "R" substituent incorrectly implies conjugation to an additional moiety. The updated graphical abstract now displays acetyl-CoA in its correct, unconjugated form.

These corrections ensure that the graphical abstract accurately reflects the biochemical mechanism of lysine acetylation and avoids potential misinterpretation.

2) Speculating on the possible effects of altered acetylation:

- The KEGG pathway enrichment analysis referenced in the sentence starting on line 594 should be included as a supplementary figure/table (with accompanying methods) and not simply listed as unpublished. It would be helpful to know what other pathways scored highly in this analysis in addition to the mitochondrial BCAA degradation pathway.

Response 5: We agree with the reviewer and have now included the complete KEGG pathway enrichment analysis as a **new supplementary figure 8**, along with the corresponding methods (Line 235-251). These additions list all enriched pathways—not only the mitochondrial BCAA degradation pathway—so readers can evaluate the full spectrum of high-scoring pathways. The Results and Methods sections have been updated accordingly.

- It would help to note that *while autophagy impacts protein acetylation patterns uniformly across subcellular compartments, the stoichiometry of these modifications differs substantially across organelles. As discussed in detail in this review (PMID: 39606008), well-established regulatory mechanisms exist for high-stoichiometry acetylation events in the nucleus, whereas the functional significance of the typically low-stoichiometry acetylation observed in mitochondria remains an active area of investigation.*

- In fitting with the point above, the sentence starting on line 596 should be changed to something like “...impaired acetylation of mitochondrial enzymes in this pathway...”

Response 6: *We appreciate the reviewer’s suggestion. The sentence has been revised to read: “...impaired acetylation of mitochondrial enzymes in this pathway...” to more accurately reflect the mitochondrial localization of BCAA-degrading enzymes and the nature of the acetylation changes. This updated wording has been incorporated into the revised manuscript.*

- Similarly, the sentence starting on line 598 should be changed to something like “...identifying **the subcellular compartments and** the specific acetylated proteins...”

Response 7: *We thank the reviewer for this helpful suggestion. The sentence has been revised to read: “...identifying the subcellular compartments and the specific acetylated proteins...” to provide clearer and more precise wording. The updated text has been incorporated into the revised manuscript.*

Minor Concerns

3) Even though it is stated later in the *Results* section, a brief explanation of why P62 was included in the immunoblots should be added when describing Figure 1 (perhaps *moving the later text* on this antibody).

Response 8: *We appreciate the reviewer’s suggestion. We have now added a brief explanation in the Figure 1 description clarifying why P62 was included in the immunoblots. Specifically, we state that “P62/SQSTM1 is a well-established marker of impaired autophagy flux, its accumulation serves as confirmation that autophagy deficiency was effectively achieved in the analyzed livers”. See red text- line 290-294*

4) line 272, it should be specified that the **analysis is for “tyrosine” phosphorylation, as serine and threonine phosphorylation are prevalent PTMs.**

Response 9: *Thank you for pointing this out. We have revised the sentence on line 272 to specify that the*

*analysis refers specifically to **tyrosine phosphorylation**, noting that serine and threonine phosphorylation are far more prevalent PTMs. This clarification has been incorporated to ensure accuracy and avoid ambiguity. See red text- line 295-297.*

5) On line 272, it should be stated that the analysis used two commercial antibodies “recognizing acetyl-lysine”, as both N-terminal acetylation and cysteine acetylation also exist.

Response 10: *We thank the reviewer for this clarification. The sentence on line 272 has been revised to specify that the analysis used **two commercial antibodies recognizing acetyl-lysine**, thereby distinguishing ϵ -lysine acetylation from other forms of acetylation such as N-terminal or cysteine acetylation. This clarification has now been incorporated into the manuscript. See red text- line 301-302.*

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Point by Point Response

Manuscript Title "Autophagy Regulates the Hepatic Acetylome Through Modulating Acetyl-CoA Metabolite"
COMMSBIO-25-5728B

Baral K et al 2026

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

The authors have addressed several of the initial concerns; however, a key mechanistic issue remains unresolved regarding the interpretation of the acetyl-CoA experiments. The proposed explanation relies on indirect hydrolysis to acetate, yet the rationale for using acetyl-CoA instead of acetate is unclear. As currently presented, this experiment does not provide clear mechanistic insight. I therefore recommend minor revision to address this point, including appropriate controls to substantiate the proposed mechanism.

Response: *We thank the reviewer for this insightful comment and for emphasizing the need for greater mechanistic clarity regarding the interpretation of the acetyl-CoA supplementation experiments. Our original rationale for administering acetyl-CoA was to determine whether replenishing an acetyl-containing metabolite could restore the reduced protein acetylation observed in autophagy-deficient livers. However, we agree that, because acetyl-CoA is generally not cell permeable, the observed effects are unlikely to result from direct intracellular uptake of acetyl-CoA.*

*To address this concern, we have revised the manuscript to clarify both the rationale and the limitations of these experiments and have moderated our interpretation accordingly (**Discussion, lines 626–642, result section- lines 539-542**). Importantly, we also evaluated the effects of acetate, a known precursor of acetyl-CoA. We found that acetate supplementation produced effects comparable to those observed with acetyl-CoA, including restoration of hepatic protein acetylation and improvement of liver pathology. These findings support the possibility that the effects of exogenously administered acetyl-CoA are mediated, at least in part, through its extracellular hydrolysis to acetate, followed by cellular uptake and reconversion to acetyl-CoA.*

Accordingly, we have revised the Discussion to emphasize that our data do not demonstrate a direct intracellular action of exogenous acetyl-CoA. Rather, they support the broader conclusion that replenishment of the acetate/acetyl-CoA pool can rescue protein acetylation and ameliorate liver injury in autophagy-deficient livers. We believe these revisions provide a more balanced interpretation of the data and address the reviewer's concern regarding the mechanistic significance of the acetyl-CoA experiments.

We appreciate the reviewer's constructive suggestion, which has helped strengthen the rigor and clarity of our study.

Reviewer #3 (Remarks to the Author):

The authors have satisfactorily addressed all my concerns. I recommend the manuscript be accepted for publication.

Response: *We sincerely thank the reviewer for the positive evaluation of our manuscript and for acknowledging that the concerns raised during the review process have been satisfactorily addressed. We appreciate the reviewer's constructive comments and valuable suggestions, which have helped improve the quality and clarity of the manuscript. We are grateful for the recommendation to accept our work for publication.*

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