nature portfolio

Peer Review File

Enzyme-Centric Chemoproteomics Reveals Isomer-Specific S-Acylation Modification Networks

Corresponding Author: Professor Suming Chen

This manuscript has been previously reviewed at another journal. This document only contains information relating to versions considered at Nature Communications.

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 manuscript by Pengfei Wu is a very interesting study. The use of metabolic labeling with unsaturated fatty acids followed by proteomics approaches to determine the unsaturated lipid substrate preference of the zDHHC enzymes for S-acylation is an interesting area of research. Indeed, the field in general knows very little regarding the lipid preferences of these enzymes. This is an important and timely study. Thank you to the authors for carefully addressing many of my prior concerns. I think the novelty of the findings are suited for Nature Communications. I have several outstanding concerns:

1. While this is the first study to combine overexpression of zDHHC enzymes with bioorthogonal labeling with unsaturated fatty acids and proteomics, the Greaves et al PNAS, 2017 paper did overexpress zDHHC enzymes and assess substrate acylation and enzyme autoacylation with various fatty acid probes, including unsaturated. Please acknowledge this in the introduction to highlight how this present study is novel from previous work.
2. The structural models with fatty acids docked were only provided for zDHHC20 and 23. All structures for which the docking was done (i.e. 20, 23, 24, 2, 7) should be provided. The fact that the protein-Ligand PDB structures were deposited to iProX is not mentioned in the appropriate section of the main text. Also, the iProX search does not appear to be working properly on the day of the review, it might be easier to just include the PDB files in the supplementary.
3. Figure 6, Line 431: “For instance, both Alk-C18:1Δ6-zDHHC20 and Alk- C18:1Δ9-zDHHC20 exhibit hydrogen bonding with H154. However, in the case of Alk-C18:1Δ11 zDHHC20, the thioester is too distant from H154 to form a hydrogen bond.” It is unclear how this conclusion was drawn because 2.2 Å is usually considered viable for hydrogen bonding. The presence of a hydrogen bond here would be more consistent with the experimental relevance of H154 shown in Figure 11.
4. Figure 6 should include C18:0 in the MM-GBSA analysis in b, c, d, e, and f. MM-GBSA values should also include units, and the graphs should have the same y-axis scale so that relative comparisons can be made.
5. It would be very helpful to perform the MM-GBSA analysis for a subset of additional zDHHC enzymes that were not experimentally analyzed. I would suggest zDHHC3/5/8/9/13/17. This may expand the findings and allow the authors and field to make broader conclusions.
6. The interpretation of the analysis in Figure 6g is focused on the hydrogen bonding with the His154 but what is likely more important when considering unsaturated fatty acids are the interactions occurring in the fatty acid acyl chain binding pocket. What are the key residues participating in interactions with the acyl chain and how is that different with the different saturation states?
7. My original concern #12 appears to have been misinterpreted, I was referring to differences in ligand affinity as measured by MM-GBSA between different zDHHCs and different fatty acids, rather than the relative activity of the enzymes. But it is still interesting that the overall structure of the binding pocket has no impact on the binding affinity. I think this raises the question of whether the residues that are responsible for fatty acid binding are conserved/different across PATs with the same/different affinity for the same fatty acid, which I don’t think figure 6 fully answers. I think it would be beneficial to include a further discussion of this in the discussion section, and a figure in the supplementary material.
8. Figure 4H, delta9+2BP condition has zero variation. Were the values normalized to this sample in each replicate? If not, why does this condition have zero variation? If a group has zero variation the assumptions of the ANOVA (that the data fit a gaussian distribution and that variances are equal between groups) are not met so a regular parametric ANOVA cannot be used to analyze these data.

9. The use of the zDHHC20 C156A and H154A variants in experiments are critical controls, thank you for including them. These controls are important because the critical role of these resides participate in catalysis is already known. However, the way the results are written makes it seem as though this is the paper that is providing this critical evidence. Please rephrase.
10. In reference to concern #13, 12 hours is still quite a long labeling time. I think the authors should acknowledge this in the discussion as a limitation. The alkyne-fatty acids may be undergoing beta-oxidation resulting in fatty acids of different chain length.

Minor comments:
1. Line 49 – DHHC should be defined (Asp-His-His-Cys)
2. Line 50 – the active site is DHHC not zDHHC as zDHHC includes the entire zinc finger
3. Line 161 refers to a Supplemental Figure 3c, which does not exist
4. Line 271 – please add the citation to the paper that did the prediction for the number of transmembrane domains for the zDHHC enzymes. Also, add the fact that these are predicted, not experimentally confirmed transmembrane domains.
5. Please add more detail in the figure legend for Fig 5c, what do all of the colours and connections mean?
6. A citation to the zDHHC20 crystal structure should be provided in line 393
7. Lines 395-400, thank you for adding this additional information regarding the rationale for using MM-GBSA. However, this detail may be better placed in the methods than the results section.
8. Line 428 should be "… we observed hydrogen bonding between …"

Reviewer #2

(Remarks to the Author)
1. The issue of novelty is my primary concern. As mentioned in the comments, the workflow bears similarity to that of previous studies on saturated fatty acids (FAs)—specifically, the combination of alkyne-tagged FA probes, click chemistry, and proteomics. The authors contend that their innovations lie in two aspects: (1) utilizing unsaturated FA isomers to investigate the effects of carbon-carbon double (C=C) bonds; and (2) integrating zDHHC overexpression with proteomics to map FA-zDHHC-substrate networks. While the focus on unsaturated FA isomers represents a meaningful extension of existing work, it does not constitute a conceptual breakthrough. This is merely an application of established chemoproteomic tools rather than the development of a new technical framework. The revised manuscript still overstates the work's novelty, and the authors need to temper their claims regarding this aspect.

2. There is no direct evidence to distinguish S-acylation from N/O-acylation for specific FA isomers. While hydroxylamine (NH_2OH) treatment confirms the prevalence of S-acylation, mass spectrometry (MS) fails to resolve which isomer modifies a specific residue. The authors have acknowledged this limitation but have not proposed any solutions beyond conventional proteomic approaches.

3. The authors justified the 12-hour metabolic labeling duration by citing relevant literature and optimizing signal intensity. However, they did not address the concern regarding probe degradation via β-oxidation. If the probe undergoes degradation, the observed lipidation events could reflect metabolites rather than the original FA isomers—this would directly undermine the method's capacity to assess isomer specificity.

4. Endogenous FA Isomer Levels in HepG2 Cells
The authors note that C18:1Δ6 is low in abundance but upregulated in cancer. However, they have not measured the endogenous levels of C18:1Δ6, C18:1Δ9, and C18:1Δ11 in HepG2 cells. If C18:1Δ6 is undetectable endogenously, the observed isomer-specific lipidation events may have limited physiological relevance.

5. Additionally, the utility of the proposed methods for other post-translational modifications (PTMs) is overstated. This study focuses exclusively on S-acylation, and no data are provided to support their broader applicability.

Reviewer #3

(Remarks to the Author)
In the revised manuscript, the authors have addressed many points raised in the initial review and have incorporated substantial new data supporting the quality of their chemoproteomic analysis. While the additional experimental efforts are acknowledged, the reviewer still has some concerns regarding the novelty and validity of the enzyme-centric chemoproteomics workflow.

1. The reviewer understands that the enzyme-centric chemoproteomics workflow differs from those used in previous related studies. However, it still remains questionable whether this workflow is fundamentally novel. FA selectivity in zDHHC family enzymes has already been investigated using clickable FA probes in combination with overexpression of several zDHHCs (PMID: 28167757). Moreover, proteome-wide identification of protein lipidation targets using clickable FA probes has been reported elsewhere. Considering these previous works, the novelty of the workflow development in the present study appears to be rather limited.

2. While the authors aim to demonstrate functional differences arising from FA isomer modifications in the context of FA β-oxidation, the experiments conducted are indirect. The authors evaluated the expression of CPT1A and DBP as β-

oxidation–related genes; however, these measurements reflect transcriptional and/or translational regulation, and it is difficult to discuss direct connection between these data and the lipidation of ACOX1 and/or ACOT8.
In terms of analysis focusing on H_2O_2, a downstream product of ACOX1, selective effect of C18:1Δ6 is interesting. Nevertheless, it remains difficult to link these findings to the lipidation of ACOX1 for the following reasons:
(i) As stated by the authors in the revised manuscript, the production of H_2O_2 is not specifically associated with ACOX1 or peroxisomal β-oxidation.
(ii) According to Figs. 3a and b, modification of ACOX1 is sensitive to NH_2OH, suggesting that it undergoes S-acylation. However, the selective increase in H_2O_2 observed upon C18:1Δ6 treatment occurs in the presence of 2BP, an inhibitor of S-acylation. Therefore, this increase appears to be unrelated to the lipidation of ACOX1.

The authors should evaluate the biological processes identified through the GO term enrichment analysis, such as β-oxidation itself or peroxisome morphology (fission), etc. Validation of these findings is essential for the manuscript to be considered for publication; without such validation, the study would remain primarily descriptive and its biological significance would be difficult to assess.

3. In supplementary Fig. 7d, the authors show that there is no significant change in the levels of lipidated proteins upon overexpression of zDHHC when using Alk-C18:0. However, given that zDHHC enzymes catalyze protein palmitoylation and that Alk-C18:0 is commonly used to detect palmitoylated proteins, capturing substrate proteins of zDHHCs with this probe should serve as a benchmark for validating enzyme-centric chemoproteomics workflow. Therefore, the absence of any significant change under these conditions raises concerns about whether the modification proteins identified using unsaturated fatty acid probes in a zDHHC-dependent manner truly represent the substrate pool of these zDHHC enzymes. The authors discuss the possibility of redundancy or compensation among different zDHHC enzymes in their response to the initial review. However, such mechanisms are generally relevant to loss-of-function contexts and are highly unlikely to apply in the present gain-of-functon(overexpression) experiments.

Other comments:
4. In Figure 3g, data for the “zDHHC20 +” condition are shown, but these results are not discussed in the manuscript.

5. In line 287, the authors mention that obtaining precise information on specific protein changes and S-acylation specificity requires the use of an expensive antibody. While this is understandable as a practical limitation, such a statement concerning financial constraints is not appropriate for an academic manuscript. The reviewer suggests that this sentence be removed.

6. As discussed in the initial review, the diversity of C=C bond positional isomers of 18:1 FA revealed by recent structural lipidomics studies is quite interesting. The reviewer suggests that these points be included in the manuscript, as they would help readers better understand the significance of this study.

7. In Fig. 5c, the authors should clarify what each color represents.

8. “Mock-transfected” would be more appropriate than “vehicle-transfected” when describing the negative control in the zDHHC overexpression experiments.

9. In line 362, the authors refer Fig. 4c, but should this actually be Fig. 5c?

10. In lines 370-373, the authors describe an amino acid substitution experiment using the C156A mutant of zDHHC20, but this section would be better placed after the covalent docking study to improve the logical flow of the manuscript.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Thank you to the authors for thoroughly addressing my prior concerns. I have two minor outstanding comments:

Line 438: "However, in the case of Alk-C18:1Δ11-zDHHC20, the thioester shows a longer distance and suboptimal angle relative to H154, consistent with a weak or absent hydrogen bond (Supplementary Fig. 17). Therefore, H154 is likely to be critical for the catalytic activity 438 of zDHHC20 in S-acylation modification". Starting the second sentence with "therefore" does not make sense here as it was just stated that Alk-C18:1Δ11-zDHHC20 does not H-bond to H154. It might be clearer to just cut out the sentence starting with therefore and just say "To test whether His154 is required for catalytic activity across different FA isomers, we mutated...”

Response to reviewer #1, comment #8: The finding that there is no high degree of binding pocket residue conservation between the different PATs that share the same FA isomer binding profile seems to be surprising and important, but the authors do not mention this in the results or discussion. In my opinion, there should be at least one sentence about this somewhere in the text, because a lot of readers will wonder the same thing.

Reviewer #2

(Remarks to the Author)
The authors claimed that the novelty of their work resides in the integration of isomer probe metabolic labeling, zDHHC overexpression, and global quantitative proteomics. However, it need to be emphasized that the latter two components are common practices in chemical proteomics and contribute little to the novelty of the study. Thus, the core innovation of this work lies in isomer probe metabolic labeling and the insights it provides regarding the impact of isomerism (C=C bond position) on the S-acylation network.
Building on this, I have carefully examined the literature cited by Reviewer 3: "FA selectivity in zDHHC family enzymes has already been investigated using clickable FA probes in combination with overexpression of several zDHHCs" (PMID: 28167757). The main difference between the two studies lies in two key aspects: the probes have shifted from focusing on "chain length variation" to "C=C isomerism," and the scientific question has expanded from "enzyme-substrates preference" to "acylation networks" (with a substantial increase in scale). As the authors acknowledged, their work did not represent a conceptual breakthrough in the technical framework, but is a new workflow that fills an existing gap.
While this is a solid study that involves systematic improvements addressing an unresolved scientific question, it is ultimately an extension of existing methods or incremental scaling in scope. I appreciate the authors' efforts in revising the manuscript, but I have to say the novelty of this work does not meet the standards required for publication in Nature Communications.

Reviewer #3

(Remarks to the Author)
In the revised manuscript, the authors have overall adequately addressed the reviewer's comments, and the new interpretations they provide appear to be generally reasonable.
One remaining concern is the functional differences arising from fatty acid isomer modifications. At present, the experimental evidence supporting these functional differences (i.e. the expression of DBP and the concentration of H_2O_2) remains indirect. It would therefore be desirable to directly evaluate the biological processes identified in Figs. 4a-c (e.g. fatty acid β-oxidation and peroxisome fission, etc.).
In the reviewer's opinion, validation of the findings obtained through chemoproteomics is crucial to avoid the study to remain primarily descriptive. However, this issue may fall outside the scope of this manuscript, and its necessity should ultimately be determined by the editors.

Response to Reviewers' Comments

Reviewer 1:

1. This manuscript by Pengfei Wu is a very interesting study. The use of metabolic labeling with unsaturated fatty acids followed by proteomics approaches to determine the unsaturated lipid substrate preference of the zDHHC enzymes for S-acylation is an interesting area of research. Indeed, the field in general knows very little regarding the lipid preferences of these enzymes. This is an important and timely study. Thank you to the authors for carefully addressing many of my prior concerns. I think the novelty of the findings are suited for *Nature Communications*.

***Reply*:** We thank the reviewer for recognizing our work and providing valuable suggestions for improving this study.

2. While this is the first study to combine overexpression of zDHHC enzymes with bioorthogonal labeling with unsaturated fatty acids and proteomics, the Greaves et al PNAS, 2017 paper did overexpress zDHHC enzymes and assess substrate acylation and enzyme autoacylation with various fatty acid probes, including unsaturated. Please acknowledge this in the introduction to highlight how this present study is novel from previous work.

***Reply*:** We thank the reviewer for this valuable suggestion. The study by Greaves et al. (*Proc. Natl. Acad. Sci. USA* 2017, 114, E1365-E1374) provides an important foundation, as it was the first to systematically examine the chain-length selectivity of zDHHC enzymes and to evaluate both substrate acylation and enzyme autoacylation using a panel of fatty-acid probes. In comparison with this seminal work, our study offers substantial deepening and extension along two dimensions:

(1) From chain length to isomerism. Greaves et al. primarily addressed zDHHC selectivity toward the chain length of saturated fatty acids (e.g., C14 vs C16). Here, we move beyond chain length and focus on the fine structural features of unsaturated fatty acids, systematically demonstrating—an aspect not previously explored—that positional isomerism of C=C double bonds can markedly shape the specificity of protein S-acylation. This provides a higher-resolution structural perspective on the molecular determinants governing lipidation.

(2) From enzyme-centric preference to proteome-wide networks. The prior study relied on zDHHC overexpression to interrogate enzyme autoacylation preferences and effects on a limited set of candidate substrates. In contrast, we integrate zDHHC overexpression with metabolic labeling using positional-isomer probes and quantitative proteomics to, for the first time, delineate proteome-scale S-acylation networks mediated by specific zDHHC enzymes in a C=C position-dependent manner (Fig. 5). This enables a conceptual shift from individual "enzyme–substrate" relationships to a systematic "enzyme–isomer–substrate" network framework.

Collectively, our study elevates zDHHC-mediated protein lipidation research to a new level that is more refined (isomer resolution), more systematic (network-scale mapping), and more comprehensive (across modification contexts). We have modified the description and discussed the gap between the previous study and our work in the revised Introduction. **Please see lines 54-59 in the manuscript.**

3. The structural models with fatty acids docked were only provided for zDHHC20 and 23. All structures for which the docking was done (i.e. 20, 23, 24, 2, 7) should be provided. The fact that the protein-Ligand PDB structures were deposited to iProX is not mentioned in the appropriate section of the main text. Also, the iProX search does

not appear to be working properly on the day of the review, it might be easier to just include the PDB files in the supplementary.

***Reply*:** Thank you for this helpful suggestion. We agree that the structural models used for docking should be made fully available to ensure transparency and reproducibility. In the revised manuscript, we have included all docking-derived PDB files as part of the Supplementary Information (**Supplementary Data 7**), covering all proteins subjected to docking analysis and we have added the corresponding information at the appropriate location

We also thank the reviewer for noting that our iProX submission was not accessible at the time of review. This issue has now been resolved, and the raw data can be accessed normally.

4. Figure 6, Line 431: "For instance, both Alk-C18:1Δ6-zDHHC20 and Alk- C18:1Δ9-zDHHC20 exhibit hydrogen bonding with H154. However, in the case of Alk-C18:1Δ11 zDHHC20, the thioester is too distant from H154 to form a hydrogen bond." It is unclear how this conclusion was drawn because 2.2 Å is usually considered viable for hydrogen bonding. The presence of a hydrogen bond here would be more consistent with the experimental relevance of H154 shown in Figure 11.

***Reply*:** Thank you for pointing out the imprecision in this key statement. Based on general structural biology principles, an H···O distance of 2.1 Å is indeed within the typical range compatible with hydrogen-bond formation (commonly ~1.5-2.5 Å). Therefore, our original statement that a hydrogen bond "cannot form" was inaccurate.

In this study, identification of hydrogen-bond interactions was primarily based on Schrödinger's Ligand Interaction analysis (**Fig. R1** blow, **Supplementary Fig. 17**). Importantly, hydrogen-bond assignment is not determined by a distance threshold alone but instead reflects combined distance and angular constraints (*J. Mol. Biol.* 1994, 238, 777-793). In the Glide scoring scheme, when the H···X distance falls within ~2.1-2.5 Å, the hydrogen bond contribution decays linearly with increasing distance to zero; similarly, as the Z–H···X angle decreases from 150° to 120°, the contribution also decays to zero (*J. Med. Chem.* 2004, 47, 1739-1749). In the Alk-C18:1Δ11–zDHHC20 complex, the N–H···O angle is only 123.7°, and under the combined effects of distance (2.1 Å) and suboptimal geometry, Schrödinger did not annotate this interaction as a hydrogen bond.

We have revised the manuscript to provide a more accurate description; please see revised manuscript **lines 436-438 on page 18**.

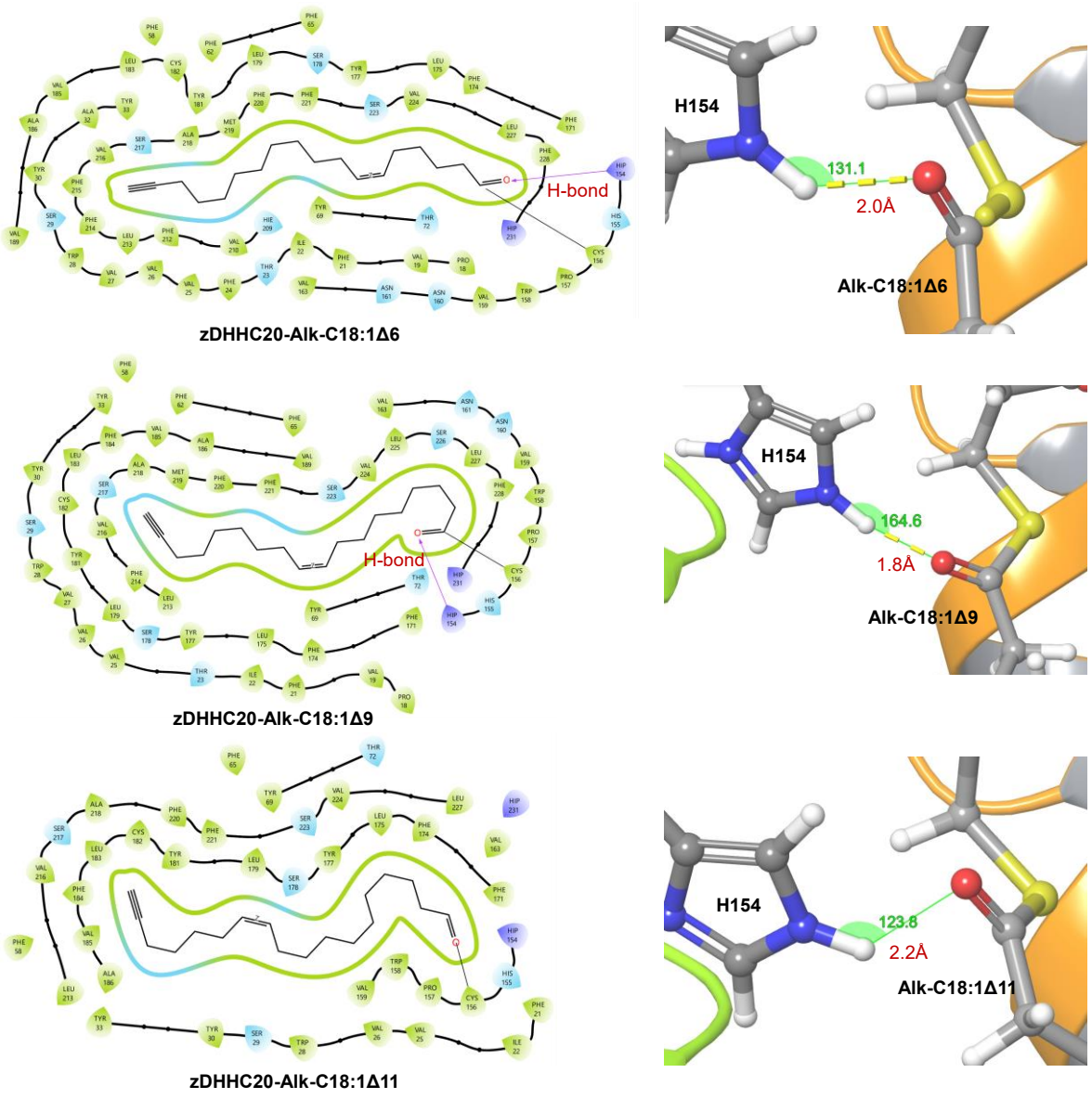

Fig. R1| Ligand interaction diagram.

5. Figure 6 should include C18:0 in the MM-GBSA analysis in b, c, d, e, and f. MM-GBSA values should also include units, and the graphs should have the same y-axis scale so that relative comparisons can be made.

***Reply*:** Thank you for the reviewer's suggestion. We have included the MM-GBSA analysis of C18:0 into the revised Figure 6 accordingly.

6. It would be very helpful to perform the MM-GBSA analysis for a subset of additional zDHHC enzymes that were not experimentally analyzed. I would suggest zDHHC3/5/8/9/13/17. This may expand the findings and allow the authors and field to make broader conclusions.

Reply: Thank you for this constructive suggestion. In response, we performed additional MM-GBSA analyses for

zDHHC enzymes that had not been experimentally characterized in our study (including zDHHC3, zDHHC5, zDHHC8, zDHHC9, zDHHC13, and zDHHC17). Overall, the MM-GBSA results indicate that the isomer-dependent binding preferences vary across different zDHHC enzymes; notably, Alk-C18:0 generally exhibits higher binding affinity toward these zDHHCs. We have added this content to the revised manuscript and Supplementary Information; please see **lines 456-460 on page 19** in the manuscript and **Supplementary Fig. 18**.

7. The interpretation of the analysis in Figure 6g is focused on the hydrogen bonding with the His154 but what is likely more important when considering unsaturated fatty acids are the interactions occurring in the fatty acid acyl chain binding pocket. What are the key residues participating in interactions with the acyl chain and how is that different with the different saturation states?

***Reply*:** Thank you for this insightful comment. In the context of unsaturated fatty acids, van der Waals interactions between the acyl chain and the enzyme binding pocket are crucial for understanding how double bond position shapes zDHHC selectivity.

Our in-depth analysis of the docking models indicates that all three C18:1 isomers make extensive van der Waals contacts with a common set of aromatic and hydrophobic residues lining the zDHHC20 hydrophobic cavity, including W158, F174, Y181, S217, and F220. These residues likely constitute a shared structural scaffold that accommodates the acyl chain. Importantly, however, differences in double bond position introduce subtle conformational changes in the acyl chain, which in turn modulate contacts with specific residues in the pocket, providing a plausible structural basis for isomer selectivity. For Alk C18:1Δ6, the more proximal double bond enables additional contacts with residues near the distal end or side wall of the cavity, including S29, L175, and V216. For Alk C18:1Δ9, the characteristic kink imposed by the Δ9 double bond leads to preferential interactions with I22 and V25 on the TM1 helix. For Alk C18:1Δ11, the more distal double bond positions the alkyne tagged tail deeper within the pocket, promoting contact with V185 on the TM3 helix.

We have incorporated these points into the revised manuscript; please see revised manuscript **lines 419-421 on page 18.**

8. My original concern #12 appears to have been misinterpreted, I was referring to differences in ligand affinity as measured by MM-GBSA between different zDHHCs and different fatty acids, rather than the relative activity of the enzymes. But it is still interesting that the overall structure of the binding pocket has no impact on the binding affinity. I think this raises the question of whether the residues that are responsible for fatty acid binding are conserved/different across PATs with the same/different affinity for the same fatty acid, which I don't think figure 6 fully answers. I think it would be beneficial to include a further discussion of this in the discussion section, and a figure in the supplementary material.

***Reply*:** Thank you for clarifying this point and for the constructive suggestion. We understand that your concern refers to differences in ligand binding affinity (MM-GBSA) across different zDHHC enzymes and fatty acids, rather than relative changes in enzymatic activity.

In the main text, we did not perform direct cross enzyme comparisons of MM-GBSA values, because MM-GBSA is most appropriately applied to rank the relative affinities of structurally similar ligands within the same receptor

(palmitoyltransferase) under a consistent structural and energetic framework (*Chem. Rev.* 2019, 119, 9478-9508). Following your suggestion, we nevertheless examined whether PATs showing similar versus distinct affinity toward the same fatty acid share conserved or divergent pocket lining residues. We compared the predicted binding modes and cavity contacting residues across these PATs. Using the Alk-C18:1Δ11 probe as an example, we first compared Δ11-zDHHC20 and Δ11-zDHHC24. Despite nearly identical MM-GBSA values (49.01 vs. 47.90), their predicted interaction profiles were largely distinct, sharing only two pocket contacting residues (W and L) among those involved in hydrogen-bonding and van der Waals contacts. Conversely, for the pair exhibiting the largest MM-GBSA difference Δ11-zDHHC2 and Δ11-zDHHC23 (53.90 vs. 10.31), we observed a higher degree of residue overlap, including four van der Waals contact residues (L, W, A, and F) and one hydrogen-bonding residue (H). Taken together, these comparisons indicate that we did not identify a consistent, rule-based residue pattern that could robustly account for the observed similarities or differences in MM-GBSA across PATs. We hypothesize that the binding of fatty acids to zDHHC enzymes is related to overall structural complementarity.

9. Figure 4H, delta9+2BP condition has zero variation. Were the values normalized to this sample in each replicate? If not, why does this condition have zero variation? If a group has zero variation the assumptions of the ANOVA (that the data fit a gaussian distribution and that variances are equal between groups) are not met so a regular parametric ANOVA cannot be used to analyze these data.

***Reply*:** Thank you for the reviewer's careful examination. Please allow us to clarify that the data points for Δ9+2BP in Figure 4h are not identical; rather, their coefficient of variation is exceptionally low compared to other groups. Their respective values are 129.3807, 129.4559, and 128.9537, reflecting variations inherent to the specific cell samples we measured.

For the quantification of H_2O_2, we normalized the measured H_2O_2 level to the total protein amount of the corresponding cell lysate, to ensure comparability across treatment groups. To avoid any potential misunderstanding, we have added a more detailed description of the raw data and the calculation procedure for Fig. 4h in the revised manuscript.

With respect to statistical testing, we agree that a conventional one way ANOVA assumes homoscedasticity. To minimize the risk of inflated type I error in the presence of unequal variances, we have therefore re analysed the dataset using Welch's ANOVA, which does not require equal variances across groups. Based on these results, we have revised the manuscript accordingly; please see the revised manuscript at **lines 233–237 on page 11**.

10. The use of the zDHHC20 C156A and H154A variants in experiments are critical controls, thank you for including them. These controls are important because the critical role of these resides participate in catalysis is already known. However, the way the results are written makes it seem as though this is the paper that is providing this critical evidence. Please rephrase.

***Reply*:** We have therefore rephrased the relevant text in the revised manuscript to more clearly acknowledge prior work. Please see the revised manuscript at **lines 444-446 on page 19.**

11. In reference to concern #13, 12 hours is still quite a long labeling time. I think the authors should acknowledge this in the discussion as a limitation. The alkyne-fatty acids may be undergoing beta-oxidation resulting in fatty

acids of different chain length.

***Reply*:** We agree that alkyne fatty acid probes may undergo intracellular metabolic conversion, including β oxidation, representing a methodological limitation of this study. Following the reviewer's suggestion, we have explicitly added and discussed this potential limitation in the end of the Discussion in the revised manuscript. Please see the revised manuscript at **lines 512–515 on page 21**.

Minor comments:

1. Line 49 – DHHC should be defined (Asp-His-His-Cys).

***Reply*:** We have revised the manuscript accordingly.

2. Line 50 – the active site is DHHC not zDHHC as zDHHC includes the entire zinc finger.

***Reply*:** This mistake has been corrected in the revised manuscript.

3. Line 161 refers to a Supplemental Figure 3c, which does not exist.

***Reply*:** We have removed the reference to "Supplementary Fig. 3c,". The text in this section describes Fig. 3d.

4. Line 271 – please add the citation to the paper that did the prediction for the number of transmembrane domains for the zDHHC enzymes. Also, add the fact that these are predicted, not experimentally confirmed transmembrane domains.

***Reply*:** We have revised the manuscript accordingly by adding the appropriate citation for the transmembrane domain prediction and by clarifying that the reported transmembrane domains are predicted rather than experimentally confirmed. Please see the revised manuscript at **lines 280–281 on page 12**.

5. Please add more detail in the figure legend for Fig 5c, what do all of the colours and connections mean?

***Reply*:** We have revised Fig. 5c accordingly by annotating the colour scheme directly in the figure and expanding the figure legend to clearly explain the meaning of the node colours and the connections. Please refer to **page 14** of the revised manuscript.

6. A citation to the zDHHC20 crystal structure should be provided in line 393.

***Reply*:** We have added the appropriate citation in the revised manuscript. Please see the revised manuscript at **line 390 on page 16**.

7. Lines 395-400, thank you for adding this additional information regarding the rationale for using MM-GBSA. However, this detail may be better placed in the methods than the results section.

***Reply*:** We have revised the manuscript accordingly.

8. Line 428 should be "… we observed hydrogen bonding between …"

***Reply*:** We have revised the manuscript accordingly.

Reviewer 2:

1. The issue of novelty is my primary concern. As mentioned in the comments, the workflow bears similarity to that of previous studies on saturated fatty acids (FAs)—specifically, the combination of alkyne-tagged FA probes, click chemistry, and proteomics. The authors contend that their innovations lie in two aspects: (1) utilizing unsaturated FA isomers to investigate the effects of carbon-carbon double (C=C) bonds; and (2) integrating zDHHC overexpression with proteomics to map FA-zDHHC-substrate networks. While the focus on unsaturated FA isomers represents a meaningful extension of existing work, it does not constitute a conceptual breakthrough. This is merely an application of established chemoproteomic tools rather than the development of a new technical framework. The revised manuscript still overstates the work's novelty, and the authors need to temper their claims regarding this aspect.

***Reply*:** Thank you for your concern and recognition of the advanced nature of our work. We agree that the technical framework proposed by our study does not represent a significant conceptual breakthrough. Nevertheless, the proposed chemical proteomics workflow integrating quantitative proteomics with enzyme overexpression still fills a gap in previous research methodologies. Previous studies combining overexpression with SDS-PAGE analysis primarily focused on the enzyme's own autoacylation preferences or a limited number of candidate substrates. This study integrates isomer probe metabolic labeling, zDHHC overexpression and global quantitative proteomics to establish a three-dimensional relational network connecting the enzyme (zDHHC), ligand structure (FA isomers), and global substrate spectrum (Figure 5c). This represents a shift from individual enzyme activity analysis to systematic network mapping.

Following your advice, we have minimized descriptions of methodological innovation in the revised manuscript, presenting it solely as a new workflow. For instance, in the Discussion section, we removed phrases such as “overcome technical challenges” and “isomer chemoproteomics technique,” while toning down descriptions of technical advantages.

2. There is no direct evidence to distinguish S-acylation from N/O-acylation for specific FA isomers. While hydroxylamine (NH_2OH) treatment confirms the prevalence of S-acylation, mass spectrometry (MS) fails to resolve which isomer modifies a specific residue. The authors have acknowledged this limitation but have not proposed any solutions beyond conventional proteomic approaches.

***Reply*:** Thank you for the reviewer’s thoughtful assessment of the limitations in resolving the modification types introduced by fatty acid isomers. In the workflow used in this study, we cannot unambiguously assign, at the single amino acid residue level, which specific C=C positional isomer, such as Δ6, Δ9, or Δ11, modifies a given site. This reflects a general and important technical bottleneck in current approaches that combine chemical biology probes with high throughput proteomics.

Despite this limitation, at the current stage of the field, the strategy we used integrates metabolic labeling, click chemistry enrichment, and hydroxylamine, NH_2OH, based selective cleavage, and it represents one of the most established and widely adopted approaches for interrogating protein S-acylation dynamics and function, for example ABE and related methods. This framework enables robust discrimination of thioester linked modifications, S acylation, from ester or amide linked modifications, N/O-acylation. It also allows modification events to be

reliably associated, at the protein level, with probes of defined structures. Within this widely accepted methodological framework, our study systematically evaluates how C=C bond position, as a fine structural feature of unsaturated fatty acids, is associated with proteome scale differences in S-acylation networks.

We agree that achieving truly isomer resolved lipidation proteomics remains an important goal for the field. To move toward this, we plan to explore complementary technical avenues in future work. For example, we will investigate tandem mass spectrometry based diagnostic fragmentation strategies by designing and synthesizing isomer encoded probes that bear cleavable tags or stable isotope labels and generate distinctive diagnostic ions. This should enable a more direct linkage between a modified site and a specific isomer in tandem mass spectra.

Thank you again for your valuable comments. We have already addressed this point in our discussion as a methodological limitation. Please see **lines 515-518** in the revised manuscript.

3. The authors justified the 12-hour metabolic labeling duration by citing relevant literature and optimizing signal intensity. However, they did not address the concern regarding probe degradation via β-oxidation. If the probe undergoes degradation, the observed lipidation events could reflect metabolites rather than the original FA isomers—this would directly undermine the method's capacity to assess isomer specificity.

***Reply*:** Thank you for raising this point. During the metabolic labeling of fatty acid probes, β-oxidation degradation is indeed a common issue. As a general guideline, metabolic labeling experiments are often recommended to within 4-16 hours to reduce fatty acid β-oxidation (*STAR Protoc.* 2025, 6, 103890; *Nat. Methods* 2012, 9, 84-89; *Cell Commun. Signal.* 2019, 17, 90). We referenced previous studies and selected a 12-hour incubation period based on metabolic labeling efficiency. During this process, β-oxidation of the probe may occur, representing a limitation of the metabolic labeling method. To ensure readers clearly understand this information, we have added a discussion on methodological limitations in the revised manuscript. Please see **lines 512–515 on page 21**.

4. Endogenous FA Isomer Levels in HepG2 Cells. The authors note that C18:1Δ6 is low in abundance but upregulated in cancer. However, they have not measured the endogenous levels of C18:1Δ6, C18:1Δ9, and C18:1Δ11 in HepG2 cells. If C18:1Δ6 is undetectable endogenously, the observed isomer-specific lipidation events may have limited physiological relevance.

***Reply*:** Thank you for the reviewer's interest in the endogenous abundance of fatty acid isomers in HepG2 cells and the physiological relevance of our observations. We agree that establishing the presence and relative abundance of unsaturated fatty acid isomers under endogenous conditions is an important prerequisite for evaluating the biological significance of isomer associated lipidation.

To address this point, we performed additional measurements of endogenous C18:1 isomer composition in HepG2 cells using a reported aziridination derivatization-based LC-MS/MS strategy (*Anal. Chem.* 2024, 96, 2524-2533) **(Fig. R2a** blow). We found that C18:1Δ6, C18:1Δ9, and C18:1Δ11 account for approximately 2.2%, 85.3%, and 12.5% of total C18:1 isomers (**Fig. R2b** blow), respectively. These data show that C18:1Δ6 is reliably detectable in HepG2 cells and present at a quantifiable endogenous fraction, rather than being absent under these conditions.

We also note that, in biological systems, certain lipid signalling mediators, such as eicosanoids, can be present at very low abundance yet exert pronounced regulatory effects through high affinity and highly specific interactions.

In this context, although C18:1Δ6 is not the predominant isomer, its isomer associated lipidation may still influence pathway output under specific conditions by selectively modulating the activity or subcellular localization of key target proteins. Consistent with this possibility, the literature we cite indicates that under stress related contexts such as SCD1 inhibition, the Δ6 desaturation route can be selectively upregulated, leading to substantial shifts in the relative abundance of C18:1Δ6 and related isomers (*Nature* 2019, 66, 403-406). This implies that, in particular physiological or pathological settings, local availability and functional contribution may differ markedly from steady state bulk measurements. The C18:1Δ6 associated lipidation network defined in our study provides a data foundation for future work aimed at delineating its potential roles during dynamic processes such as metabolic reprogramming.

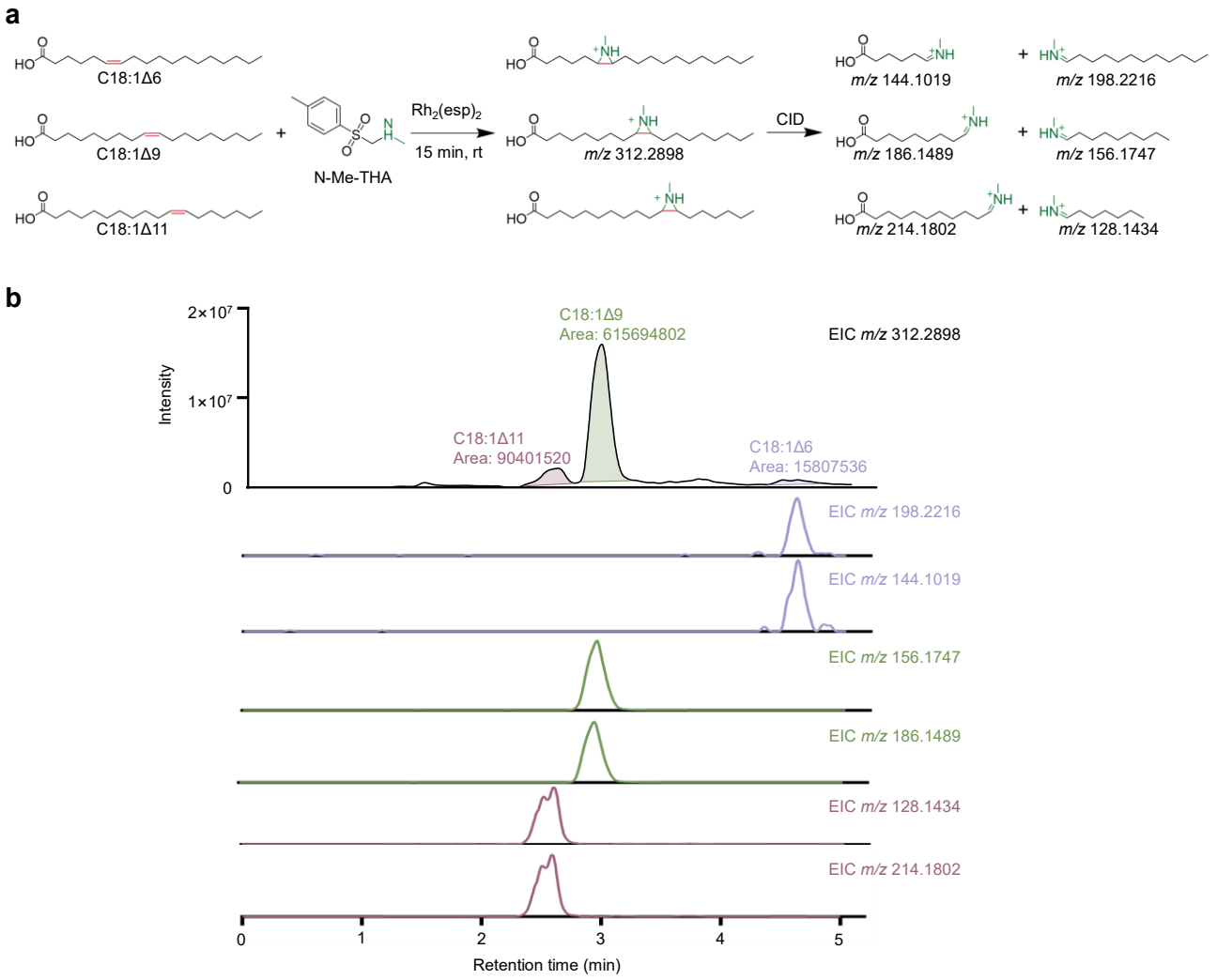

Fig. R2| Quantification of the proportions of fatty acid isomers in HepG2 cells.

5. Additionally, the utility of the proposed methods for other post-translational modifications (PTMs) is overstated. This study focuses exclusively on S-acylation, and no data are provided to support their broader applicability.

***Reply*:** We have deleted the statements on applicability to other PTMs in the revised manuscript.

Reviewer 3:

1. The reviewer understands that the enzyme-centric chemoproteomics workflow differs from those used in previous related studies. However, it still remains questionable whether this workflow is fundamentally novel. FA selectivity in zDHHC family enzymes has already been investigated using clickable FA probes in combination with overexpression of several zDHHCs (PMID: 28167757). Moreover, proteome-wide identification of protein lipidation targets using clickable FA probes has been reported elsewhere. Considering these previous works, the novelty of the workflow development in the present study appears to be rather limited.

***Reply*:** Thank you for your detailed comments on the novelty of our study. We agree that the technical framework proposed by our study does not represent a significant conceptual breakthrough. Nevertheless, the chemical proteomics workflow integrating quantitative proteomics with enzyme overexpression still fills a gap in previous research methodologies. Previous studies combining overexpression with SDS-PAGE analysis primarily focused on the enzyme's own autoacylation preferences or a limited number of candidate substrates. This study integrates isomer probe metabolic labeling, zDHHC overexpression and global quantitative proteomics to establish a three-dimensional relational network connecting the enzyme (zDHHC), ligand structure (FA isomers), and global substrate spectrum (Figure 5c). This represents a shift from individual enzyme activity analysis to systematic network mapping. Specifically, we would highlight three aspects in which our study extends previous work:

Shift and refinement of the central question, from chain length to fine structural features of unsaturation. The seminal study you cited (*Proc. Natl. Acad. Sci.* USA 2017, 114, E1365-E1374) established zDHHC selectivity with respect to fatty acyl chain length. Building on this foundation, our study extends the question to a distinct structural parameter of unsaturated fatty acids, namely the positional isomerism of the C=C bond. Because C=C position can alter acyl chain geometry, conformational preferences, and steric fit within hydrophobic environments, interrogating this variable requires position defined isomer probes and enables questions that are not directly addressed by chain length comparisons. Here we carried out a systematic evaluation of C=C positional isomer effects in this context.

Integration of the experimental design, from enzyme preference or candidate substrate validation to proteome scale network mapping. Prior work combining overexpression with clickable fatty acid probes has primarily aimed to define enzyme level acyl donor preferences, including autoacylation, and to validate a limited number of candidate substrates. In contrast, we integrate isomer probes, a multi zDHHC overexpression panel, and quantitative proteomics to move beyond enzyme preference alone and to summarize, at the proteome scale, the S-acylation substrate landscapes associated with specific zDHHC enzymes under defined C=C isomer conditions (Fig. 5c). This analysis provides a global view of how fine fatty acid structural features, in a given enzyme context, are associated with distinct cellular modification patterns.

New biological discoveries and mechanistic insights. Leveraging the unique integrated approach described above, we were able to report novel findings that would be difficult to obtain solely through studies limited to a small number of candidate substrates:1) At the proteome scale, our data indicate that C=C bond position is associated with distinct S-acylation profiles (Fig. 3e). 2) We further observe that the same zDHHC enzyme can be associated with markedly different substrate patterns across FA isomers, supporting a role for acyl chain type in shaping

zDHHC-dependent substrate selectivity (Fig. 5c, d-f). 3) Finally, we link isomer-associated differences to variations in zDHHC autoacylation intermediate abundance (Fig. 6b-f) and to differences in inferred interactions within the substrate binding pocket from docking analyses (Fig. 6g-h), providing initial mechanistic clues for the observed selectivity.

2. While the authors aim to demonstrate functional differences arising from FA isomer modifications in the context of FA β-oxidation, the experiments conducted are indirect. The authors evaluated the expression of CPT1A and DBP as β-oxidation–related genes; however, these measurements reflect transcriptional and/or translational regulation, and it is difficult to discuss direct connection between these data and the lipidation of ACOX1 and/or ACOT8.

In terms of analysis focusing on H_2O_2, a downstream product of ACOX1, selective effect of C18:1Δ6 is interesting. Nevertheless, it remains difficult to link these findings to the lipidation of ACOX1 for the following reasons:

(i) As stated by the authors in the revised manuscript, the production of H_2O_2 is not specifically associated with ACOX1 or peroxisomal β-oxidation.

(ii) According to Figs. 3a and b, modification of ACOX1 is sensitive to NH_2OH, suggesting that it undergoes S-acylation. However, the selective increase in H_2O_2 observed upon C18:1Δ6 treatment occurs in the presence of 2BP, an inhibitor of S-acylation. Therefore, this increase appears to be unrelated to the lipidation of ACOX1.

The authors should evaluate the biological processes identified through the GO term enrichment analysis, such as β-oxidation itself or peroxisome morphology (fission), etc. Validation of these findings is essential for the manuscript to be considered for publication; without such validation, the study would remain primarily descriptive and its biological significance would be difficult to assess.

***Reply*:** Thank you for these incisive and important comments on the functional validation of fatty acid isomer effects. We agree with your central concern. Although in Fig. 4e-g and the associated experiments we measured changes in CPT1A and DBP protein abundance and monitored metabolic readouts such as cellular H_2O_2, these readouts primarily reflect expression or steady state changes and overall metabolic status. At present, they do not establish a direct causal link between the specific isomer dependent lipidation events on ACOX1 or ACOT8 and the observed phenotypes.

Your comments prompted us to reevaluate our interpretation of the Fig. 4 results. Based on your critique and our additional analyses, we have revised our conclusions and will comprehensively update the corresponding text in the manuscript. Specifically, under conditions in which global S-acylation is inhibited by 2-BP, supplementation with C18:1Δ6, but not Δ9 or Δ11, selectively increased DBP protein abundance and cellular H_2O_2. This pattern suggests that the observed effect is unlikely to be mediated by S-acylation. In the same setting, we also observed increased modification of ACOT8 **(Fig. R3 blow, Supplementary Fig. 5**), which we had identified as a protein undergoing Δ6 associated N/O-acylation. We therefore propose an alternative working hypothesis: C18:1Δ6 may influence ACOT8 function by enhancing its N/O-acylation, a modification not inhibited by 2-BP, thereby altering the peroxisomal β-oxidation flux and ultimately leading to increased DBP expression and elevated H_2O_2 production. We emphasize that this remains a hypothesis supported by multi omics associations and preliminary observations. Nevertheless, it links the phenotype to a specific candidate molecular event, namely Δ6 associated N/O-acylation of ACOT8, and

provides a clearer basis for targeted follow up experiments. You can find our updated conclusions in **lines 261–270 on page 12** of the returned revised manuscript.

We would like to clarify that the primary goal and central advance of this study is the proteome scale, systematic identification of pronounced specificity of unsaturated fatty acids differing only in C=C bond position within S acylation networks (Figs. 3 and 5), together with mechanistic and enzymatic insights into this specificity (Fig. 6). The functional experiments in Fig. 4 were designed to provide initial, hypothesis generating biological context for this broad modification landscape. They were not intended to fully delineate a specific pathway, but rather to suggest that 1) distinct isomer associated modification networks are accompanied by distinct functional signatures, as supported by GO enrichment analyses, and 2) the Δ6 associated network shows a notable connection to peroxisomal metabolism that warrants further investigation.

Overall, the key advance of this work is the upstream mapping of isomer associated modification specificity, whereas the functional data provide preliminary clues and testable hypotheses regarding its potential biological consequences.

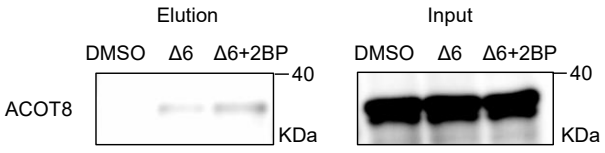

Fig. R3 | Western blot analysis of the effect of 2-BP on ACOT8 lipidation.

3. In supplementary Fig. 7d, the authors show that there is no significant change in the levels of lipidated proteins upon overexpression of zDHHC when using Alk-C18:0. However, given that zDHHC enzymes catalyze protein palmitoylation and that Alk-C18:0 is commonly used to detect palmitoylated proteins, capturing substrate proteins of zDHHCs with this probe should serve as a benchmark for validating enzyme-centric chemoproteomics workflow. Therefore, the absence of any significant change under these conditions raises concerns about whether the modification proteins identified using unsaturated fatty acid probes in a zDHHC-dependent manner truly represent the substrate pool of these zDHHC enzymes. The authors discuss the possibility of redundancy or compensation among different zDHHC enzymes in their response to the initial review. However, such mechanisms are generally relevant to loss-of-function contexts and are highly unlikely to apply in the present gain-of-functon(overexpression) experiments.

***Reply*:** Thank you for your careful analysis of the data in Supplementary Fig. 7d and for raising this key point. We fully understand your concern. In a gain of function overexpression setting, the lack of a clear global increase in labeling with the canonical palmitoylation probe Alk C18:0 requires a reasonable explanation.

We agree that, in an overexpression model, invoking functional redundancy as a generic explanation for a negative result is not fully satisfying. Based on further consideration and analysis, we think this observation is more likely to reflect different sensitivities of a high background steady state network versus a lower background, more selective network to perturbation by a single zDHHC enzyme. This difference arises from the intrinsic properties of the two

regimes.

For saturated fatty acids such as C18:0, Alk C18:0 labels the largest number of S acylated proteins in the basal state, approximately 630 proteins (Fig. 3c), indicating that the endogenous zDHHC network already uses this probe efficiently and that the overall modification flux may be close to the steady state capacity of this cellular system. This network is also likely supported by multiple zDHHC enzymes with substantial substrate overlap. Under these conditions, overexpression of any single enzyme, for example zDHHC20, would be expected to make only a small marginal contribution relative to the large basal flux. Such a modest increment can be difficult to detect against a high background and may not reach the statistical thresholds used in our proteomics analysis (S_0 = 2, FDR = 0.01). Thus, the result does not necessarily indicate the absence of any effect, but rather that any increase is diluted by the high basal signal and falls below our detection sensitivity.

By contrast, for unsaturated fatty acid positional isomers, the number of labeled S-acylated proteins at baseline is lower, with 539, 581, and 551 proteins for C18:1Δ6, C18:1Δ9, and C18:1Δ11, respectively (Fig. 3c), and each isomer shows a substantial set of unique substrates (Fig. 3e). This pattern is consistent with the idea that these modifications are driven by a smaller subset of zDHHC enzymes with more selective roles. In this setting, overexpression of a given zDHHC can produce larger relative changes in substrate acylation that are more readily detectable.

We also appreciate your suggestion to use Alk C18:0 as a positive control for the overexpression system. In our view, the catalytically inactive mutant zDHHC20 C156A provides a more stringent and direct control (Supplementary Fig. 11). Overexpression of this mutant abolished the signal increases observed with all probes, including Alk C18:0. This demonstrates that (a) the overexpression system is functional and (b) any observed enhancement effects strictly depend on zDHHC catalytic activity.

We thank you again for this insightful question, which prompted us to articulate a more general biological interpretation and strengthened the logic of our study.

Other comments:

4. In Figure 3g, data for the “zDHHC20 +” condition are shown, but these results are not discussed in the manuscript.

Reply**:** Thank you for your careful review. We have added a description of “zDHHC +” in the revised manuscript. Please see **lines 310–312**.

5. In line 287, the authors mention that obtaining precise information on specific protein changes and S-acylation specificity requires the use of an expensive antibody. While this is understandable as a practical limitation, such a statement concerning financial constraints is not appropriate for an academic manuscript. The reviewer suggests that this sentence be removed.

Reply**:** We have removed this sentence from the revised manuscript.

6. As discussed in the initial review, the diversity of C=C bond positional isomers of 18:1 FA revealed by recent structural lipidomics studies is quite interesting. The reviewer suggests that these points be included in the

manuscript, as they would help readers better understand the significance of this study.

***Reply*:** Thank you for this suggestion. We have added this discussion of the diversity of C=C bond positional isomers of 18:1 fatty acids revealed by recent structural lipidomics studies to the revised manuscript. Please see **lines 62–67 on page 2**.

7. In Fig. 5c, the authors should clarify what each color represents.

***Reply*:** We have revised Fig. 5c accordingly by annotating the colour scheme directly in the figure and expanding the figure legend to clearly explain the meaning of the node colours and the connections.

8. “Mock-transfected” would be more appropriate than “vehicle-transfected” when describing the negative control in the zDHHC overexpression experiments.

***Reply*:** We have revised this description in the revised manuscript.

9. In line 362, the authors refer Fig. 4c, but should this actually be Fig. 5c?

***Reply*:** Thank you for pointing this out. You are correct that this citation was an error. We have corrected this in the revised manuscript.

10. In lines 370-373, the authors describe an amino acid substitution experiment using the C156A mutant of zDHHC20, but this section would be better placed after the covalent docking study to improve the logical flow of the manuscript.

***Reply*:** Thank you for this suggestion. We have moved this section to follow the covalent docking analysis in the revised manuscript. Please see **lines 444–446 on page 19**.

Response to Reviewers' Comments

Reviewer 1:

Thank you to the authors for thoroughly addressing my prior concerns. I have two minor outstanding comments: Line 438: "However, in the case of Alk-C18:1Δ11-zDHHC20, the thioester shows a longer distance and suboptimal angle relative to H154, consistent with a weak or absent hydrogen bond (Supplementary Fig. 17). Therefore, H154 is likely to be critical for the catalytic activity 438 of zDHHC20 in S-acylation modification". Starting the second sentence with "therefore" does not make sense here as it was just stated that Alk-C18:1Δ11-zDHHC20 does not H-bond to H154. It might be clearer to just cut out the sentence starting with therefore and just say "To test whether His154 is required for catalytic activity across different FA isomers, we mutated..."

***Reply*:** Thank you for the reviewer's suggestion. We have revised the manuscript accordingly. Please see **lines 366–368**.

Response to reviewer #1, comment #8: The finding that there is no high degree of binding pocket residue conservation between the different PATs that share the same FA isomer binding profile seems to be surprising and important, but the authors do not mention this in the results or discussion. In my opinion, there should be at least one sentence about this somewhere in the text, because a lot of readers will wonder the same thing.

***Reply*:** We thank the reviewer for this important suggestion. We agree that the limited conservation of residues associated with similar FA isomer binding profiles warrants mention. Accordingly, in the revised manuscript we have added a sentence discussing this point. Please see **lines 344–346**.

Reviewer 2:

The authors claimed that the novelty of their work resides in the integration of isomer probe metabolic labeling, zDHHC overexpression, and global quantitative proteomics. However, it needs to be emphasized that the latter two components are common practices in chemical proteomics and contribute little to the novelty of the study. Thus, the core innovation of this work lies in isomer probe metabolic labeling and the insights it provides regarding the impact of isomerism (C=C bond position) on the S-acylation network.

Building on this, I have carefully examined the literature cited by Reviewer 3: "FA selectivity in zDHHC family enzymes has already been investigated using clickable FA probes in combination with overexpression of several zDHHCs" (PMID: 28167757). The main difference between the two studies lies in two key aspects: the probes have shifted from focusing on "chain length variation" to "C=C isomerism," and the scientific question has expanded from "enzyme-substrates preference" to "acylation networks" (with a substantial increase in scale). As the authors acknowledged, their work did not represent a conceptual breakthrough in the technical framework, but is a new workflow that fills an existing gap.

While this is a solid study that involves systematic improvements addressing an unresolved scientific question, it is ultimately an extension of existing methods or incremental scaling in scope. I appreciate the authors' efforts in revising the manuscript, but I have to say the novelty of this work does not meet the standards required for publication in Nature Communications.

***Reply*:** Thank you for your meticulous review and insightful comments on this study. We understand and respect your core feedback regarding innovation. You accurately point out that the core innovation of this study lies in the metabolic labeling of isomer probes and its revelation of the impact of C=C bond positions on the S-acylation network. The proposed technique combining zDHHC overexpression with quantitative proteomics indeed fills a gap in current research methodologies. We have carefully reflected on your suggestions and wish to further elaborate on the potential deeper contributions of this work to reassess its alignment with the journal's scope:

1. From technological application to the unveiling of new biological dimensions. We contend that a study's fundamental innovation should not be measured solely by whether it invented a conceptually groundbreaking new tool, but rather comprehensively evaluated based on the novel biological knowledge revealed by the new method and its advancement of field understanding. Greaves et al.'s work established chain length as a key dimension of zDHHC enzyme selectivity. This study, however, introduces a novel, finer-grained structural parameter (C=C bond position) to the field for the first time. It systematically demonstrates that this parameter can independently reshape the topological structure and functional output of the entire S-acylation modification network at the proteome scale, independent of chain length. This represents not merely a refinement of existing dimensions, but the revelation of a previously unrecognized structural code layer in lipidation regulation. In this sense, our work pioneers a new research direction for understanding the precise regulation of protein lipidation.

2. Paradigm shift from enzyme-substrate pairs to system network maps. You accurately note that this study expands the scientific inquiry from enzyme-substrate preferences to acylation networks. We contend this represents not merely an expansion of scale, but a paradigm shift in research. Previous studies focused on enzyme preference for specific fatty acids and enzyme-substrate specificity. This represents a point-to-point paradigm. By integrating multi-

enzyme overexpression, an isomer probe library, and global proteomics, our study has for the first time constructed a three-dimensional relational network linking enzymes (zDHHC) – FA isomers – global substrate profiles (Figure 5c). This represents a leap from point-to-point enzyme activity analysis to system-wide network mapping. The revealed network provides a novel perspective on how cells coordinate complex, differentiated functional outputs through subtle modifications of FA fine structures using limited enzymatic resources.

3. Preliminary elucidation of novel biological mechanisms. Building upon this systematic expansion, we proposed and preliminarily validated a novel mechanistic hypothesis: the abundance of zDHHC enzyme autoacylation intermediates, coupled with their specific van der Waals interaction networks with different isomers within hydrophobic pockets, collectively form the molecular basis for isomer selectivity (Figure 6). This discovery links enzymatic mechanisms, FA fine structures, and downstream substrate networks, offering deeper structural and mechanistic insights into the specific regulation of lipidation.

We humbly accept your critique but respectfully request you reconsider this study's contributions from the perspective of novel biological knowledge. It establishes C=C positional isomerism of unsaturated fatty acids as a core regulatory parameter in the protein S-acylation network for the first time, and reveals at the systems level how this parameter shapes differentiated modification landscapes and functions through specific enzymes. We believe this discovery opens a new research dimension in the field and holds promise for extensive connections with future applications in disease mechanisms, drug development, and other areas.

Reviewer 3:

1. In the revised manuscript, the authors have overall adequately addressed the reviewer's comments, and the new interpretations they provide appear to be generally reasonable.

One remaining concern is the functional differences arising from fatty acid isomer modifications. At present, the experimental evidence supporting these functional differences (i.e. the expression of DBP and the concentration of H2O2) remains indirect. It would therefore be desirable to directly evaluate the biological processes identified in Figs. 4a-c (e.g. fatty acid β-oxidation and peroxisome fission, etc.).

In the reviewer's opinion, validation of the findings obtained through chemoproteomics is crucial to avoid the study to remain primarily descriptive. However, this issue may fall outside the scope of this manuscript, and its necessity should ultimately be determined by the editors.

***Reply*:** Thank you for your renewed review of the revised manuscript and your overall recognition of this research. You noted that our evidence for functional differential validation remains indirect and suggested directly assessing the biological processes identified in Figures 4a-c (e.g., fatty acid β-oxidation, peroxisome fission). At the same time, you fairly pointed out that this issue may fall outside the scope of this manuscript, and its necessity should be ultimately determined by the editor.

We agree with your assessment of the importance of functional validation. Establishing direct causal links between newly identified chemically modified events and precise biological functions would undoubtedly enhance the depth and impact of this research. However, as you astutely observe, this indeed constitutes a distinct and systematic research phase, often requiring years of effort and integration of multidisciplinary approaches to complete. Such work undoubtedly forms an independent and comprehensive research topic, extending beyond the core objective of this paper, which is to discover and characterize isomer-specific modification networks.

Therefore, we respectfully request that the Editor and Reviewers consider: The core contribution of this study lies in the first systematic revelation of the C=C bond position as a novel regulatory dimension within the protein S-acylation network, alongside the omics-level mapping of isomer-structure-dependent modification patterns mediated by specific zDHHC enzymes. This discovery itself holds independent scientific value, opening new research avenues in the field. The in-depth analysis of functional mechanisms represents a natural extension and future challenge within this new direction, rather than a prerequisite that must be fulfilled in the current manuscript.

We are willing to selectively address the limitations of current functional experiments more explicitly in the Discussion section and outline future validation directions based on the editor's decision. Alternatively, if the current functional data is deemed insufficient, we can consider moving it to supplementary materials to focus the paper more narrowly on mapping the modification network and preliminary exploration of its mechanisms.

Regardless of the final decision, we sincerely appreciate the profound insights and constructive feedback you provided throughout the review process, which significantly enhanced the rigor and scientific merit of this study.