nature portfolio

Peer Review File

Genetically encoding ε-N-methacryllysine into proteins in live cells

Corresponding Author: Professor Bing Yang

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

Attachments originally included by the reviewers as part of their assessment can be found at the end of this file.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

In the submitted manuscript, Zu et al. describe the identification of posttranslational methacrylation on K125 within cytosolic Cyclophilin A. To investigate the effect of this posttranslational modification, the authors utilize genetic code expansion to genetically encode e-N-Methacryllysine (MeaK). They confirm that MeaK can be encoded in HEK293T cells and choose to closely investigate the effect of methacrylation on Cyclophilin A, which they link to increasing cellular ROS levels through a series of experiments. They also investigate which lysine deacylase may be responsible for the removal of MeaK, generally utilizing a GFP reporter and specifically for Cyclophilin A. Generally, sirtuins and HDAC are responsible for the removal of MeaK, consistent with previous reports identifying Sirtuin 1 and 2 in this role. For MeaK Cyclophilin A, they show HDAC1 is able to remove K125MeaK when overexpressed. Finally, they provide some evidence that Kmea may be methylated in cells.

Overall, the authors present a range of different experiments utilizing genetic code expansion to encode MeaK and its potential biological effects. However, the manuscript is quite dense and often lacks proper experimental description, making it difficult to follow. Particularly, the lack of proper figure descriptions makes understanding their findings very difficult; descriptions of supplementary figures are often only a single generic sentence. The manuscript lacks a clear line of thought as some experiments are not followed up on and interpretation is left to the reader. Given these general points, I would not recommend the manuscript for publication in its present form as it appears very unrefined and still has severe gaps.

I further have summarized some more specific concerns below:

Figure 1:
• Panel b lacks any description other than that the table contains TXN1. It is unclear why it was included in the main figure.
• Panel e: A blot for CrotK should be included as the MS signal would be the same if it is a mixture of Kmea and Kcr. It's unclear to what extent the used antibody (details not given) can discriminate between Kmea and Kcr. They should confirm the absence of Kcr.
• The authors' argument could be further strengthened by demonstrating the signal's response to exogenously added methacrylate and crotonate.
• Panel H should come before f and g.

Figure 2:

• 2b: The claim that the mutants of MbPylRS used were “rationally designed” by the authors is false. The listed mutants (SI Figures 2 and 3) are all known mutants of Mm or MbPylRS which have been tested here for MeaK encoding. For example, MeaKRS1 is a transplant of MmXYRS, a variant for encoding phenylalanine derivatives. There is no rational reason to assume it would encode MeaK, suggesting that known PylRS variants were simply tested. This in itself is a reasonable approach; however, it is unclear why the authors decided to construct a false narrative. Needless to say, this is unacceptable.
• A MbPylRS variant for Acryllysine exists and has not been tested. This would be the rational choice, which is Mb-PylRS D76G, L266M, L270I, Y271F, L274A, C313F. https://doi.org/10.1002/anie.201303477

• The mutation Y384F used for MeaK encoding is a known mutation found to enhance PylRS activity with a wide range of substrates. The Y384F mutation confers no specificity to a given substrate as suggested by renaming it to MeaKRS4; this should be corrected. 10.1016/j.chembiol.2008.10.004 The respective mutation in MbPylRS Y349F has been reported to have the same effect; it also encodes Kcr lysine and is not specific to MeaK https://doi.org/10.1039/C9OB00992B. The authors should therefore definitively prove that exclusively MeaK is encoded and not Kcr (due to cellular interconversion or impurities), considering that these cannot be distinguished otherwise, which would impact all downstream experiments.
• 2b is unreadable; both positions for Mb and MmPylRS should be given here and the SI Figures.
• Non-natural amino acids should be changed to non-canonical or non-standard amino acids as MeaK occurs naturally.
• Panel c: No figure description, organism given for tRNA, unclear what protein MBP-Z is. Ideally, encoding efficiency is tested with a GFP reporter in comparison to a known substrate such as AllocK or BocK and the wtMbPylRS.
• SI Figure 3: The authors claim that MeaKRS8 was derived from chPheRS-1, however, the SI figure states MbPylRS as the source and mutations are given for MmPylRS. Given the mutation, it is MmPylRS(Y384F) and chPheRS-1 was never used? tRNA used are not stated. chPheRS-1 also is not known to encode lysine derivatives as derived from a phenylalanine tRNA-synthetase, it is unclear how the given mutations would relate to the chPheRS sequence.
• Panels F and G are redundant.
• Position 302 is alanine in MmPylRS (SI figure 2).

Figure 3:
• The relevance of panels A and B is not clear. Why is acetylation important in this context?
• This experiment seems to be disconnected from the rest of the manuscript. None of the hits are followed up on nor are they discussed further.
• It is unclear whether this effect is specific to MeaK or whether Kcr would have the same effect. The proteomic analysis should compare the two to each other.
• They should highlight HDAC1-3 as they mention this later in the manuscript.
• The authors make claims on the binding affinity of some hits; however, this is not supported by any of their experiments.
• The red asterisk in d is not explained. The low amount of CypA K125MeaK precipitated hinders a quantitative comparison with the other CypA variants. Again, a better comparison would be between MeaK and Kcr variants.

Figure 4:

• Figure description is very sparse.
• The question of whether this effect is specific to MeaK remains unanswered, or how this would correlate with any previous experiments such as the IP performed in Figure 3.
• SI Figure 9 shows the expression of GFP, not CypA.
• Panel E description is insufficient to understand their findings, the role of CsA is not explained.
• Panels are mislabelled in the figure description.

Figure 5:

• Labeling should be eGFP K85TAG for the reporter.
• Panel A: Should list NAM and NaBu.
• Panel B: Normalized signal of GFP to RFP should be shown rather than the separate values for GFP and RFP. Each cell signal should be individually quantified for GFP and normalized to its internal RFP control. Number of cells quantified is not given, and it is not clear from the methods how the analysis was done.
• Panel C: No proper description, none of these proteins are shown in Figure 3 or mentioned as significant hits.
• Panel D: Why was CypA125MeaK omitted in the pull-down with HDAC2? This is not an appropriate control and should be done in the same way as for HDAC1/3. Panel E: An in vitro assay may be better suited to determine the activity of Sirtuins and HDACs for Kmea for CypA specifically, as has been previously done for Histone Kmea: https://doi.org/10.1038/s41421-021-00344-4.
• What fraction of expressed CypA-125 is acylated in the absence of HDAC1? Given that the authors show unspecific deacylation of GFP, this should also occur with CypA to some extent?
• It is unclear to how this experiment is suited to identify the erasing deacylase considering that all cellular deacylases are present.
• HDAC1 is also not usually localized in the cytosol under normal circumstances. The authors do not further discuss the biological implication of this finding or whether this is just a consequence of their experimental setup.
•
Figure 6:

• This is an interesting observation in line with previous reports; however, it is not further discussed. The authors may consider moving this figure to the SI.
• Panel G should be moved before E and F.

Discussion

- Disconnected from the rest of the manuscript, this is the first time TDP-43 is mentioned.
- Does not cover most observation in any way

Reviewer #2

(Remarks to the Author)
Review to the manuscript entitled „Genetically encoding e-N-methacryllysine into proteins in live cells" submitted by Zhu and co-workers

Zhu et al. submitted a manuscript describing the site-specific incorporation of methacryl-lysine (Kmea) into proteins using a synthetically evolved aminoacyl-tRNA-synthetase/tRNACUA pair based on the PylRS/PylT from Methanosarcina barkeri. The authors describe the first non-histone, Cyclophilin A, to be the first protein being identified as lysine-methacrylated. The authors incorporated Kmea in a EGFP reported protein and into CypA and analyzed the impact on protein function. Using Kmea-modified EGFP and CypA they identified mammalian classical deacetylases as potent to reverse lysine-methacrylation. The authors performed mass-spectrometry to identify interactions being impaired or obtained dependent on the presence of methacrylation at two lysine side chains, i.e. K125 and K131. Finally, the authors describe Kmea being further modified by methylation to yield N-( )-methyl-N-( )-methacryllysine (Kmemea). Overall, it is an interesting study, however, in its current form I cannot recommend publication in Nature Communications. Find the points of criticism in the following section:

1. The text is written very superficial in almost all sections. The discussion does not contain any information on regulation of Kmea/Kmemea in cells. It Kmemea also removed by classical deacetylases or sirtuins? How is the protein Kmea-ylated In the first place. Are there enzymes, i.e. acyltransferases, or is it all non-enzymatically catalyzed? Is there methacryl-CoA in cells? If yes, how is it generated, in which concentrations is it present and in which organelles/subcellular localization do you find it?What is the stoichiometry of Kmea at K125 and/or K131 in CypA? Is that sufficient to generate a physiologically important effect? This should be discussed.
2. The figure legends do not contain sufficient information to understand what is shown. Several figures in the main text and the Supplementary Information file are not corresponding to the figures and panels within the figures. Please check this carefully. The lenges must contain all essential information to be able to understand what is shown.
3. How was Kmea-ylation identified in CypA? Was that by MS on the endogenous protein level? Pelase explain this more precisely.
4. How is Kmemea generated from Kmea? Is there a methyltransferase? If it is no known, please discuss this.
5. CypA was previously shown to be acetylated and the impact of acetylation was reported. Does Kmea or Kmemea also affect CsA binding? If it affects CsA binding it can be speculated that it also affects substrate binding. How is the catalytic activity affected by Kmea/Kmemea at K125 or K131?
6. Which antibody did the authors use to detect Kmea? It is specific for Kmea or does it also detect Kcr? Please provide data showing the antibody is specific.
7. The authors describe they used the crosslinkers BVSB and PDES to crosslink CypA. How was that experimentally done? Didi they overexpress CypA? Please add this information.
8. The peptides the authos show to be crosslinked in Fig. 1a suggest that there is a simple disulfide bond formed. Can the authors clarify this.
9. Fig. 1 b shows the table of identified CypA interaction partners. Can the authors explain why they decided to study thioredoxin? There were only two unique peptides identified, which is much less as observed for SPTSH and NCOR1.
10. The authors suggest interaction of CypA and thioredoxin, Kmea-ylation affecting the affinity of the interaction. How did they conclude this? A first indication would be if they analyzed the interaction in vitro by performing a protein-protein interaction study by SPR, ITC, MST or similar.
11. Fig. 1e. It is not mentioned which antibody is used. How often was the experiment performed. Can the quantify the blot?
12. The authors describe identification of Kmea by LC-MS/MS due to formation of an cyclic immonium ion similar as was described for lactylation. Did the authors detect Kmea at all in the peptides or was the detection purely done by generation of the cyclic immonium ion? Did the authors also detect the linear immonium ion? I think for lactylation it was detected.
13. Fig. 2b. How was the Y349 selected as a residue being critical for the improved PylRS variant to charge the tRNA with methacryllysine? Can the authors also give numbers how much more efficient the Y349F variant was compared to PylRS? The figure is almost not readable. Please prepare a figure that shows clearly what you want to show.
14. For the recombinant expression of MBP-Z they used a different PylRS variant compared to the cellular studies later. Can the authors explain why that was necessary?
15. How specific are the improved PylRS variants for Kmea charging the cognate tRNA and the subsequent incorporation? Do the enzymes not incorporate any other amino acid. PylRS is rather unspecific and it results in incorporation of other amino acids as well.
16. Microscopy images: almost all microscopy images are not visible and it is a really hard task to see what one should see. Please provide improved images.
17. Can you provide a ESI-MS molecular weight determination of the whole MBP-Z protein? That will show if the protein is quantitatively Kmea-ylated. For EGFP-Y15Kmea (sometimes the authors write Kmea, sometimes MeaK, please be consistent) the authors also detect EGFP-Y15K protein (Fig. 1h). Can the authors explain how this was occurring? Does the improved PylRS-variant also charge PylT with K?
18. For some peptides, such as in Fig. 1i, you write "U" in the fragmentation spectrum. Does this mean there is a Kmea and in the peptides, the authors write a "K", that there is a K and the Kmea can only be identified by the cyclic immonium ion? Please clarify. In some figures, such as Fig. 3c, there is no cyclic immonium ion shown. Why is there no cyclic immonium ion?
19. The authors consistently write "upregulated"/"downregulated" for the MS-IP data. This is wrong. The authors do not perform gene expression studies. It only means that they either identified proteins interaction stronger with one variant or better with another variant. In that context it does not become clear why the authors only focused on the improved interactions, while they did not say anything about proteins, which bind stronger to Cyp WT compares to the Kmea-ylated

proteins.
20. It would be interesting to discuss how a Kmea-ylation would differently regulate CypA function compared to acetylation or other acylations. Can the authors discuss this.
21. Fig. 3d. Information is missing where the nanobody comes from (also for the image in the Supplementary information file).
22. In Fig. 3b and Supp. Fig. 6 the authors describe the observation that in selected tumor cells acetylation at K125 and K131 is stronger or reduced compared to the non-acytlated protein. The pattern is different for K131 and K125. Can the authors provide a mechanism that underlies these differences. The authors do not give a real reference for the data, they derived the hypothesis from.
23. Fig. S5. Which antibody was used? If you stain with anti-Kmea antibody and with anti-Cyp-antibody the authors would habe a loading control.
24. In the MS-data in Fig. S7 and S8 the level is lower for Kmea-ylated protein compared to CypA K125A and WT. Is the data corrected for different protein abundances? In fif. S8 an SDS-PAGE is provided, in Fig. S7 not, is there a reason? In Fig. S8b the nanobody band is not labelled. Legend of Fig. S8 does not correspond to the figure.
25. Fig. S9, S10, S11 is only one single sentence. This is not sufficient information to understand what is shown. The cell images are almost invisible.
26. Fig. S12. The SIRT2 blot suggests unspecific binding.
27. In Fig. 5a the authors write “NAM” to indicate an inhibition of deacetylases, later they only use butyrate. Importantly, nicotinamide is a non-competitive inhibitor only for sirtuins and butryrate is a quite low-potency inhibitor only for classical deacetylases. The authors suggest CypA being deacetylated by both enzyme classes. Why did they not include NAM and butyrate. As a suggestion, SAHA and TSA are also pan-HDAC inhibitors, however, with a much higher potency in the nanomolar range compared to butyrate.
28. It is not clear why the authors used the technique with the self-cleavable peptide. This needs a better description.
29. Fig. 5d. The assay format for HDAC2 is different from HDAC1 and HDAC3. It cannot be concluded if HDAC2 is capable to act as deacetylase. The experiment must be repeated.
30. Fig. 5b, bar graphs: Can the authors explain the labelling of the y-axes. Is that relative EGFP fluorescence?
31. Fig. 4. The axes lack units.
32. Fig. S2: a loading control is missing. The legend is rudimental.
33. Fig. S3: another enzyme was generated. Why was that done?
34. Several typos are in the manuscript and the writing is not correct (Fig. S9: “Incorporate MEaK…”; better: “Incorporation of….”; “western” with capital letter, i.e. “Western”;
35. Line 156: “Stronger binding affinity was detected”. How was that determined?
36. Avoid using abbreviations such as “weren’t” in line 185.
37. The authors present a model according to which Kmea-ylatio of CypA affects ROS production by interfering with CsA binding. Did the authors experimentally measure if Kmea-ylation of CypA affects CsA binding?
38. Wording, Line 87,88: “…..of cells and reduce its target proteins wih conserved CXXC motif”. It is of course not the motif itself reducing the target proteins. Please rewrite this.
39. Line 138: “…but it was mutated to methionine….”. What do the authors want to say here? There is a different residue at this position due to the selection pressure during evolution. Can the authors say how the enzyme was phylogenetically developed? Otherwise they cannot make this statement.
40. What are “single-chain construct of CypA” (line 178), please explain.
41. Please carefully check if all necessary reference

Reviewer #3

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

Reviewer #4

(Remarks to the Author)
The paper entitled “Genetically encoding ε-N-methacryllysine into proteins in live cells” revealed a novel post-translational modification Kmea on a non-histone protein CypA. Besides, the authors developed a general genetic code expansion approach to incorporate an unnatural amino acid (Uaa) ε-N-Methacryllysine (MeaK) into target proteins and identified interacting proteins of methacrylated CypA using affinity-purification MS, by which they found that Kmea at CypA site 125 regulated cellular redox homeostasis and HDAC1 is the regulator of Kmea on CypA. Overall, this paper is written logically. The finding is interesting and meaningful. The conclusions can be supported by their data. This paper can be further improved by addressing some concerns.

1. The authors should clarify their rationale to discuss thioredoxin at the beginning of their story because no background regarding the relationship between Kmea and thioredoxin or redox process was introduced in the introduction.
2. Is CypA the only protein undergoes Kmea modification? If so, the authors should provide an explanation to this unusual finding. IF not, they should include more kmea proteins and explain why they choose to focus on CypA.
3. The authors identified HDAC1 as the eraser of CypA Kmea, which is convincing. How about the writers and readers? Moreover, HDAC1-3 has significant functional similarities and all of them can interact with CypA K125MeaK, why only HDAC1 can regulate CypA Kmea but HDAC2-3 cannot? Please add a few lines to discuss this.

4. In this paper, the authors used DCFH-DA method to determine ROS level. Although DCFH-DA is a universe method, however, this is not specific to detect hydrogen peroxide, superoxide anion or hydroxyl radicals. Please perform more specific assays to detect cellular ROS.
5. The function of CypA Kmea is not clear. As the author used HeLa, a cancer cell line as a cellular model, how does CypA Kmea regulate cancer hallmarks, such as proliferation, metastasis, drug resistance, immune escape, metabolism alteration, or cell death? The authors should at least choose one hallmark to further investigate.
6. Throughout the paper, the conclusions are all based on in vitro evidence, which is not compelling. Please perform in vivo model to strengthen this manuscript. For example, an orthotopic mouse model or a PDX mouse model. They should verity their main findings in an in vivo model, including the CypA Kmea modification, the HDAC1 as the eraser of CypA Kmea. To this end, a HDAC1 knockout mouse model is suitable for elucidate that.
7. The authors found that Kmea on CypA decreases the antioxidant ability of CypA, which is interesting. What is the mechanism underlying Kmea-mediated functional change of CypA? Please perform mechanistic experiments or add a few lines to discuss this.
8. The authors have to add some clinic relevance to this manuscript. For example, does CypA Kmea exhibit different level between normal and cancerous tissues? Does CypA Kmea hold prognostic value in cancer patients? Is CypA Kmea site (125) frequently mutated in clinic sample?
9. In this paper, the authors found that CypA Kmea can be further methylated (Kacme), which is interesting. They have to clarify the functional differences between Kmea and Kacme on CypA. Given that Kmea significantly regulates the antioxidant capability of CypA as the authors found, what is the impact of Kacme on CypA antioxidant function?

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The revision has significantly improved the quality of the manuscript. The authors should address the following remaining issues prior to publication:
- The tRNA used should be stated alongside the Synthase for each Experiment.

- chPylRS reference still refers to chPheRS paper, which was not used according to the authors. The reference should be corrected to Bryson D.
et al. Nature Chemical Biology, 2017 as stated in the review.

- Spelling of Cyclosporin(e) is inconsistent.

- Conclusions made to PRDX1-6 are poorly supported.

- General language editing is strongly advised.

Reviewer #2

(Remarks to the Author)

Reviewer #3

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

Reviewer #4

(Remarks to the Author)
The authors have addressed most points and made the manuscript easier to understand. Therefore, I consider the paper acceptable for publication.

Version 2:

Reviewer comments:

Reviewer #2

(Remarks to the Author)

Attached

Reviewer #1 (Black: reviewer comments, Blue: our response)
In the submitted manuscript, Zu et al. describe the identification of posttranslational methacrylation on K125 within cytosolic Cyclophilin A. To investigate the effect of this posttranslational modification, the authors utilize genetic code expansion to genetically encode e-N-Methacryllysine (MeaK). They confirm that MeaK can be encoded in HEK293T cells and choose to closely investigate the effect of methacrylation on Cyclophilin A, which they link to increasing cellular ROS levels through a series of experiments. They also investigate which lysine deacylase may be responsible for the removal of MeaK, generally utilizing a GFP reporter and specifically for Cyclophilin A. Generally, sirtuins and HDAC are responsible for the removal of MeaK, consistent with previous reports identifying Sirtuin 1 and 2 in this role. For MeaK Cyclophilin A, they show HDAC1 is able to remove K125MeaK when overexpressed. Finally, they provide some evidence that Kmea may be methylated in cells.

Overall, the authors present a range of different experiments utilizing genetic code expansion to encode MeaK and its potential biological effects. However, the manuscript is quite dense and often lacks proper experimental description, making it difficult to follow. Particularly, the lack of proper figure descriptions makes understanding their findings very difficult; descriptions of supplementary figures are often only a single generic sentence. The manuscript lacks a clear line of thought as some experiments are not followed up on and interpretation is left to the reader. Given these general points, I would not recommend the manuscript for publication in its present form as it appears very unrefined and still has severe gaps.

I further have summarized some more specific concerns below:

Figure 1:
• Panel b lacks any description other than that the table contains TXN1. It is unclear why it was included in the main figure.
Response: We apologize for causing confusion. We have clarified it in the figure legend.

• Panel e: A blot for CrotK should be included as the MS signal would be the same if it is a mixture of Kmea and Kcr. It's unclear to what extent the used antibody (details not given) can discriminate between Kmea and Kcr. They should confirm the absence of Kcr.
Response: We thank the reviewer for the his/her comments. The pan specific anti-Kmea antibody was purchased from PTM BioLab, Inc. (Catalog No. PTM-1501). According to the paper (Delaney K. et al. Cell Discovery, 2021), this anti-Kmea antibody has 20-fold more specific binding activity for methylacrylylated peptides than for crotonylated peptides. We also repeated the binding experiment using synthetic peptides and found that the anti-Kmea antibody didn't recognize Kcr, although the anti-Kcr antibody showed cross-reactivity (Figure R1a). We treated cells with sodium methacrylate or sodium crotonate. The Kmea signal of CypA increased with the increasing doses of sodium methacrylate (Figure R1b). Similarly, the Kcr signal of CypA also increased with the increasing doses of sodium crotonate (Figure R1b). Therefore, it is likely that CypA was both methacrylated and

crotonylated. A total of 9 Kcr sites on CypA have been identified by large-scale proteomics analysis (Yu H. et al. Science Advances, 2020).

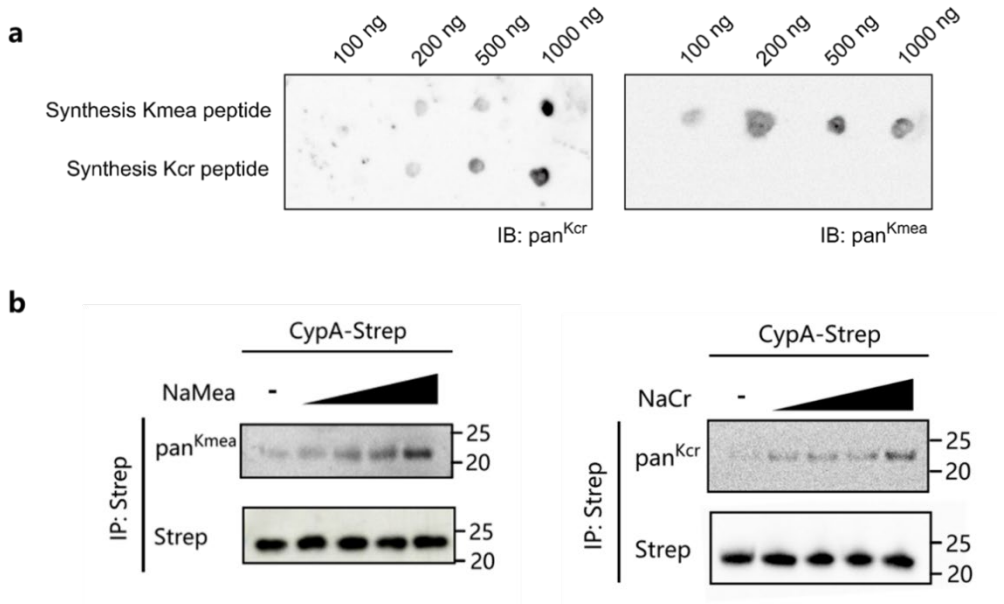

Figure R1 (Supplementary Figure 1). Specificity of the commercial Kmea antibody and Kcr antibody (a) Synthesized peptides HVVFGKmeaVK and HVVFGKcrVK were used to assess the specificity of pan anti-Kmea and anti-Kcr antibody in Dot blot assay. The amounts of peptides spotted in each column of the membrane were labeled on the top of figures. (b) HEK293T cells expressing Strep-tagged CypA were treated with 1, 3, 5 and 10 mM sodium methacrylate (NaMea) or sodium crotonate (NaCr) for 24 h, separately. Strep-tag enriched CypA was analyzed by Western blot.

• The authors' argument could be further strengthened by demonstrating the signal's response to exogenously added methacrylate and crotonate.
Response: We thank the reviewer for his/her comments. We have treated HEK 293T cells with different concentrations of exogenous methacrylate and crotonate, and validated that CypA was methacrylated and crotonylated (Figure R1b).

• Panel H should come before f and g.
Response: We thank the reviewer for pointing this out. We have changed the order of the panels.

Figure 2:

• 2b: The claim that the mutants of MbPylRS used were “rationally designed” by the authors is false. The listed mutants (SI Figures 2 and 3) are all known mutants of Mm or MbPylRS which have been tested here for MeaK encoding. For example, MeaKRS1 is a transplant of MmXYRS, a variant for encoding phenylalanine derivatives. There is no rational reason to assume it would encode MeaK, suggesting that known PylRS variants were simply tested. This in itself is a reasonable approach; however, it is unclear why the authors decided to construct a false narrative. Needless to say, this is unacceptable.

Response: We thank the reviewer for his/her comments. We have changed this sentence “based on rational design” to “based on published protein structure”. We do design these mutants based on docking MeaK to the protein structure of tRNA synthetase, except MmXYRS. We trie MmXYRS because this synthetase has high Uaa incorporation efficiency and specificity.

• A MbPylRS variant for Acryllysine exists and has not been tested. This would be the rational choice, which is Mb-PylRS D76G, L266M, L270I, Y271F, L274A, C313F. https://doi.org/10.1002/anie.201303477

Response: We thank the reviewer for his/her comments. This paper (https://doi.org/10.1002/anie.201303477) is very interesting, it enlightens us to fluorescent labeling Kmea modification in cells in the future. We found that AcrKRS do recognize Kmea, but its incorporation efficiency is relative lower than *Mb*PylRS Y349F. (Figure R2).

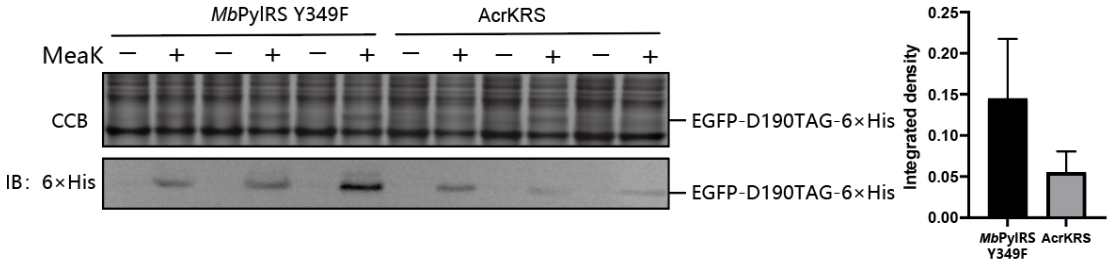

Figure R2. Western blot analysis to evaluate the MeaK incorporation efficiency on EGFP with *Mb*PylRS mutant. The loading control was shown by Coomassie brilliant blue (CBB) staining. The intensity of EGFP was normalized by loading control. Three biological replicates were performed.

• The mutation Y384F used for MeaK encoding is a known mutation found to enhance PylRS activity with a wide range of substrates. The Y384F mutation confers no specificity to a given substrate as suggested by renaming it to MeaKRS4; this should be corrected. 10.1016/j.chembiol.2008.10.004 The respective mutation in MbPylRS Y349F has been reported to have the same effect; it also encodes Kcr lysine and is not specific to MeaK https://doi.org/10.1039/C9OB00992B. The authors should therefore definitively prove that exclusively MeaK is encoded and not Kcr (due to cellular interconversion or impurities), considering that these cannot be distinguished otherwise, which would impact all downstream experiments.

Response: We thank the reviewer for his/her comments. We have replaced the ‘MeaKRS4’ with ‘*Mb*PylRS (Y349F)‘. We agree with the reviewer that *Mb*PylRS (Y349F) have wide range of substrates. However, we have not found any report about cellular interconversion of MeaK to crotonyllysine. We have strict quality control on the chemical synthesis of MeaK to ensure no crotonyllysine impurities. Intact protein analysis and tandem mass spectrometry measurement have shown that no contaminant Uaa was incorporated into target proteins.

• 2b is unreadable; both positions for Mb and MmPylRS should be given here and the SI

Figures.

Response: We thank the reviewer for his/her comments. We have labelled both *Mb*PylRS and *Mm*PylRS positions.

• Non-natural amino acids should be changed to non-canonical or non-standard amino acids as MeaK occurs naturally.

Response: We thank the reviewer for pointing this out. We have replaced all of 'unnatural amino acids' with 'non-canonical amino acids'.

• Panel c: No figure description, organism given for tRNA, unclear what protein MBP-Z is. Ideally, encoding efficiency is tested with a GFP reporter in comparison to a known substrate such as AllocK or BocK and the wtMbPylRS.

Response: We thank the reviewer for his/her comments. We have rewritten the legend of Fig. 2c and explained the construct MBP-Z. The tRNA is from *Methanosarcina barkeri*. We have labeled it in the main text. We used the EGFP report for comparison with BocK to test coding efficiency. (Figure R3)

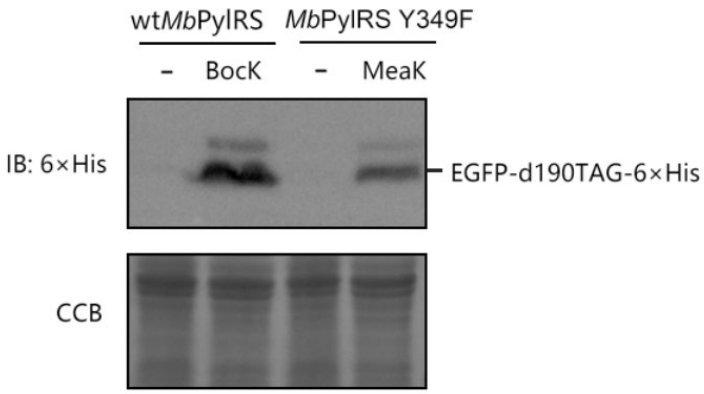

Figure R3. The Western blot on the top shows amber suppression with and without MeaK in comparison to Boc-lysine (BocK). The loading control was *E. coli* whole cell lysates analyzed by Coomassie brilliant blue (CBB) stained SDS-PAGE gel.

• SI Figure 3: The authors claim that MeaKRS8 was derived from chPheRS-1, however, the SI figure states MbPylRS as the source and mutations are given for MmPylRS. Given the mutation, it is MmPylRS(Y384F) and chPheRS-1 was never used? tRNA used are not stated. chPheRS-1 also is not known to encode lysine derivatives as derived from a phenylalanine tRNA-synthetase, it is unclear how the given mutations would relate to the chPheRS sequence.

Response: We apologize for the mistake. For the mammalian cell experiments,synthetase chPylRS was used. It contains amino aicds 1-148 of *Mb*PylRS and amino aicd 149-418 of *Mm*PylRS (Bryson D. et al. Nature Chemical Biology, 2017). The orthogonal tRNA is *M. mazei* $tRNA^{Pyl}$. The sequence of tRNA can be found in figure1A of this paper. (https://doi.org/10.1093/nar/gkx1156)

• Panels F and G are redundant.

Response: We thank the reviewer for his/her comments. In our last published paper (Liu DD. et al. Nature Communication, 2024), the reviewer required us to add the panel like Fig. 2f.

• Position 302 is alanine in MmPylRS (SI figure 2).
Response: We apologize for this mistake. We have corrected it.

Figure 3:
• The relevance of panels A and B is not clear. Why is acetylation important in this context?
Response: We apologize for the confusion. For panels A and B, we want to show the importance of site CypA Lys125. Panel A shows that Lys125 is conserved in various species, the conserved sites are often important for protein functions. Panel B show that acetylation level of CypA Lys125 is increased in tissue of lung adenocarcinoma. We can not do this kind of analysis on Kmea due to limited data of Kmea have been published. Actually, we carried out immunohistochemical analysis on normal and lung cancer tissues of human and mouse. Unfortunately, the pan specific anti-Kmea antibody is not suitable for immunohistochemical analysis, so we haven't shown these data in the manuscript. We show the acetylation data in panel B, because the acylations have similar substrates, catalytic enzymes, biological function and related diseases (Xu Y. et al. Molecular & Cellular Proteomics).

• This experiment seems to be disconnected from the rest of the manuscript. None of the hits are followed up on nor are they discussed further.
Response: We thank the reviewer for his/her comments. In CypA IP experiments, we found that CypA K125MeaK have stronger interaction with proteins involved in redox homeostasis compared with CypA K125A and WT. Then we focused on that CypA K125MeaK regulated ROS level of cells. HDAC1-3 were identified in CypA K125MeaK IP and we carried out Co-IP experiments to validate interactions between CypA K125MeaK and HDAC1-3. We revealed that HDAC1 may de-methacrylate CypA.

• It is unclear whether this effect is specific to MeaK or whether Kcr would have the same effect. The proteomic analysis should compare the two to each other.
Response: We thank the reviewer for his/her comment. We performed comparison between MeaK and Kcr insertions at CypA site 125(Figure R4a). The IP proteome of the Kcr and MeaK insertions, which overlap by approximately 75% (Figure R4b). In biological processes, the unique proteins of CypA-125MeaK IP are mainly enriched in nucleic acid processing and cell differentiation (Figure R4c). Compared to Kcr at the CypA125 site, the presence of Kmea would likely also affect cell differentiation and enhancement of hits related to the cGAS/STING and PI3K/AKT signaling pathway (Figure R4d).

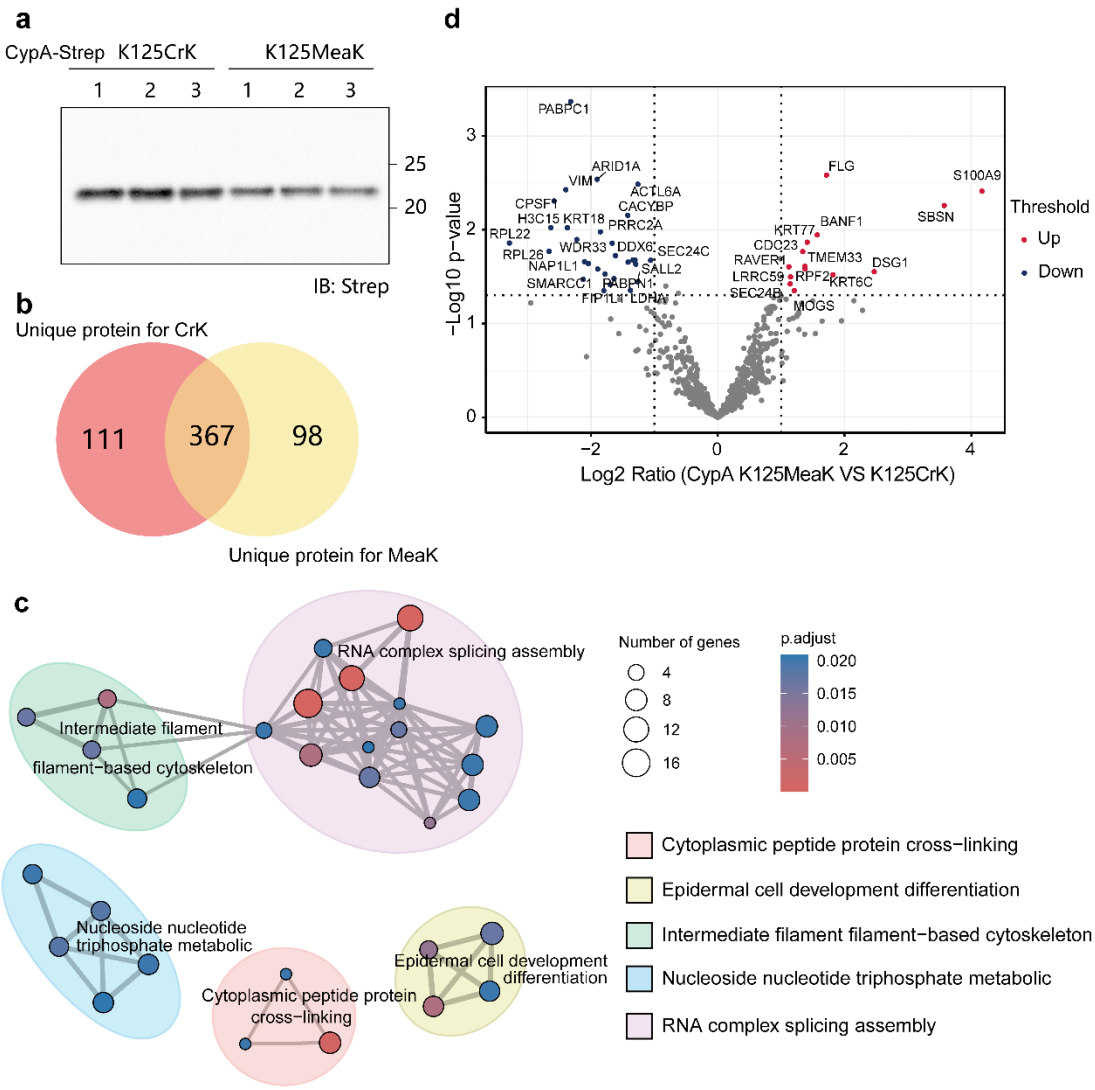

Figure R4 (Supplementary Figure 9). Identify interacting proteins of CypA (K125MeaK, K125Kcr). (a) Western blot of CypA (K125MeaK, K125Kcr) Strep-IP (three biological replicates). (b) Venn diagram showing the interactome of CypA K125MeaK and CypA K125Kcr. (c) Biological process analysis of the unique proteins identified in CypA K125Meak in Strep-IP. (d) Volcano plot of semi-quantitation on CypA K125MeaK and CypA K125Kcr IP samples. Red dots, significantly upregulated hits; Blue dots, significantly downregulated hits. Grey dots, non-differentially hits. (Fold Change ≥ 2, p-value < 0.05).

• They should highlight HDAC1-3 as they mention this later in the manuscript.

Response: We thank the reviewer for his/her comment. We have discussed HDAC1-3 in the manuscript

• The authors make claims on the binding affinity of some hits; however, this is not supported by any of their experiments.

Response: We thank the reviewer for his/her comments. We have changed these sentences ‘has stronger binding affinity’ to ‘has stronger interactions’.

• The red asterisk in d is not explained. The low amount of CypA K125MeaK precipitated hinders a quantitative comparison with the other CypA variants. Again, a better comparison would be between MeaK and Kcr variants.
Response: We thank the reviewer for his/her comments. The red asterisks mark the positions of CypA. Our revision on comparing the effects of MeaK and Kcr has been responded to previously (Figure R4).

Figure 4:

• Figure description is very sparse.
Response: We thank the reviewer for pointing this out. We have rewritten the description of this figure.

• The question of whether this effect is specific to MeaK remains unanswered, or how this would correlate with any previous experiments such as the IP performed in Figure 3.
Response: We thank the reviewer for his/her comments. Peroxiredoxins are members of peroxidases which can reduce peroxides. CypA was reported to bind with perodiredoxin-6 (PRDX6) and enhance antioxidant activity of PRDX1-6 (Lee S. et al. The Journal of Biological Chemistry, 2001). In our CypA IP experiments, intensity of PRDX4 in CypA K125MeaK is 3.5 fold less than intensity of PRDX4 in CypA K125A IP (without normalizing intensity of PRDX4). There is no significant change on intensity of PRDX4 in CypA K125MeaK IP and CypA WT IP. These data suggest that Kmea of CypA may perturb interaction between CypA and PRDX4 and suppress antioxidant activity of CypA. In Figure 3, they are regular IP experiments, we cannot differentiate which binding proteins have direct interactions with CypA. Now, we have synthesized photo-crosslinkable MeaK (xMeaK), the direct interacting proteins of CypA can be identified by mapping xMeaK mediated cross-linking peptides. In the future, we will apply xMeaK to further study how methacrylated CypA regulate ROS level of cells.

• SI Figure 9 shows the expression of GFP, not CypA.
Response: We apologize for this mistake. We have corrected it.

• Panel E description is insufficient to understand their findings, the role of CsA is not explained.
Response: We thank the reviewer for pointing this out. We have corrected it.

• Panels are mislabelled in the figure description.
Response: We apologize for this mistake. We have corrected it.

Figure 5:

• Labeling should be eGFP K85TAG for the reporter.
Response: We thank the reviewer for pointing this out. We have corrected it.

• Panel A: Should list NAM and NaBu.
Response: We apologize for this mistake; we have corrected it.

• Panel B: Normalized signal of GFP to RFP should be shown rather than the separate values for GFP and RFP. Each cell signal should be individually quantified for GFP and normalized to its internal RFP control. Number of cells quantified is not given, and it is not clear from the methods how the analysis was done.
Response: We thank the reviewer for his/her comments. In this experiment, we are unable to give an accurate positive cells number, which relies on flow cytometry. We have re-analyzed the experiment data again. As shown in Figure R5, we measured the total intensity of the GFP and the RFP signal separately using ImageJ and normalized the GFP signal to the RFP signal for each group. The GFP signal was dependent on the presence of MeaK. Adding NaBu or NAM can suppress the GFP signal significantly.

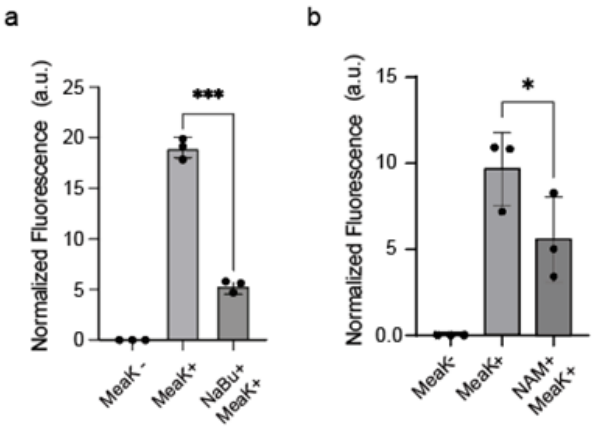

Figure R5. Detection of EFGP K85MeaK and mCherry fluorescence in live cells after treating cells with deacetylase inhibitors. (a) Genetically encoded Kmea can be removed in live cells treated with 10mM NaBu. (b) Genetically encoded Kmea can be removed in live cells treated with 10mM NAM. n = 3 independent experiments. *, $p < 0.05$; ***, $p < 0.001$; (two-tailed t-test analysis).

• Panel C: No proper description, none of these proteins are shown in Figure 3 or mentioned as significant hits.
Response: HDAC1-3 are not significant hits in Figure 3, but they are only binders of CypA with known deacetylase activities. Therefore, they were considered as putative 'erasers' of Kmea. CypA is a highly acylated protein with 9 published acetylation sites and 9 Kcr sites. Therefore, CypA WT and CypA K125A may also bind HDAC 1-3. There is no significant difference of HDAC 1-3 levels in CypA K125Meak, CypA WT and CypA K125A IP experiments.

• Panel D: Why was CypA125MeaK omitted in the pull-down with HDAC2? This is not an appropriate control and should be done in the same way as for HDAC1/3. Panel E: An in vitro assay may be better suited to determine the activity of Sirtuins and HDACs for Kmea for CypA specifically, as has been previously done for Histone Kmea: https://doi.org/10.1038/s41421-021-00344-4.

Response: We thank the reviewer for his/her comments. We have updated this panel in Figure 4. The *in vitro* de-methacylation experiments we also performed, and it was shown that HDAC1 can catalyze the removal of Kmea *in vitro* (Figure R6).

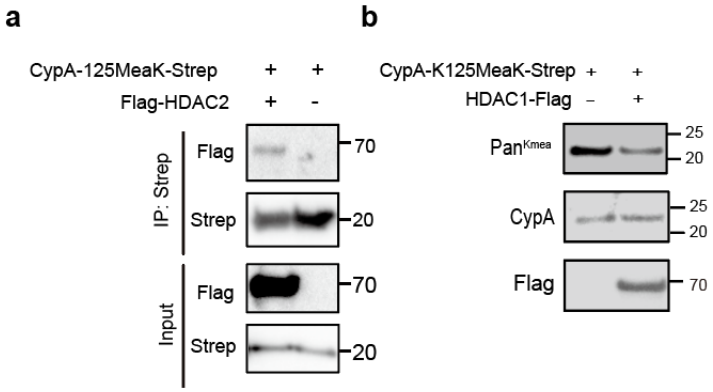

Figure R6 (Figure 5e and g). Confirmation of interaction between CypA K125MeaK and HDAC2 and *in vitro* de-methacrylation assay of HDAC1. (a) Validation of interactions between CypA K125MeaK and HDAC2 by Co-IP experiments. (b) CypA K125MeaK and HDAC1 were purified from HEK293T cells. CypA K125MeaK was incubated with or without recombinant HDAC1 for 2h at 30 °C, and then samples were analyzed by Western blot.

• What fraction of expressed CypA-125 is acylated in the absence of HDAC1? Given that the authors show unspecific deacylation of GFP, this should also occur with CypA to some extent?

Response: We thank the reviewer for his/her comments. In mammalian cells, the de-acylases have broad selectivity. They can recognize different type of acylations. One acylation site was often regulated by multiple de-acylases. We show that HDAC1 is one of de-methacrylases. There should be other enzymes also capable of de-methacrylation.

• It is unclear to how this experiment is suited to identify the erasing deacylase considering that all cellular deacylases are present.

Response: We thank the reviewer for his/her comments. We have performed *in vitro* de-methacrylation experiment to validate the de-methacrylase (Figure R6).

• HDAC1 is also not usually localized in the cytosol under normal circumstances. The authors do not further discuss the biological implication of this finding or whether this is just a consequence of their experimental setup.

Response: We thank the reviewer for his/her comments. Although HDAC1 localizes only in the nucleus, CypA also has nuclear localization. There are various cytosol and nuclear localized proteins that have been reported to be deacylated by HDAC1, such as NR1D2, RELA, SP1 and STAT3, most of which are transcriptionally associated. Similarly, as described in the discussion section of manuscript, the function of nuclear localized CypA is associated with the assembly of heterogeneous nuclear ribonucleoprotein complexes of TDB-43 in the nucleus.

Figure 6:

• This is an interesting observation in line with previous reports; however, it is not further discussed. The authors may consider moving this figure to the SI.
Response: We thank the reviewer for his/her comments. In this manuscript, we have confirmed that Kmea can be further methylated to Kmemea in cells. We thought this could be an interesting finding for the protein acylation research community. Future research effort will be involved in identifying endogenous sites, writers, erasers and readers of Kmemea.

• Panel G should be moved before E and F.
Response: We have moved Panel G before Panel E and F.

Discussion

- Disconnected from the rest of the manuscript, this is the first time TDP-43 is mentioned.
Response: 'CD147, TDP-43, HNRNPA1 HNRNPA2B1' are well-known interactors of CypA. They were also identified in our CypA experiments, we want to show that our IP experiments are robust.

- Does not cover most observation in any way
Response: We thank the reviewer to pointing this out. We have further discussed about Kmemea modifications.

Reviewer #2 (Black: reviewer comments, Blue: our response)

Review to the manuscript entitled „Genetically encoding e-N-methacryllysine into proteins in live cells" submitted by Zhu and co-workers

Zhu et al. submitted a manuscript describing the site-specific incorporation of methacryl-lysine (Kmea) into proteins using a synthetically evolved aminoacyl-tRNA-synthetase/tRNACUA pair based on the PylRS/PylT from Methanosarcina barkeri. The authors describe the first non-histone, Cyclophilin A, to be the first protein being identified as lysine-methacrylated. The authors incorporated Kmea in a EGFP reported protein and into CypA and analyzed the impact on protein function. Using Kmea-modified EGFP and CypA they identified mammalian classical deacetylases as potent to reverse lysine-methacrylation. The authors performed mass-spectrometry to identify interactions being impaired or obtained dependent on the presence of methacrylation at two lysine side chains, i.e. K125 and K131. Finally, the authors describe Kmea being further modified by methylation to yield N-(□)-methyl-N-(□)-methacryllysine (Kmemea). Overall, it is an interesting study, however, in its current form I cannot recommend publication in Nature Communications. Find the points of criticism in the following section:

1. The text is written very superficial in almost all sections. The discussion does not contain any information on regulation of Kmea/Kmemea in cells. It Kmemea also removed by classical deacetylases or sirtuins?

Response: We thank the reviewer for his/her comments. We have shown that HDAC1 is responsible for removal of Kmea. For Kmemea, we are generating pan-specific Kmemea antibodies with peptide library, but it is challenging to get high specificity Kmemea antibodies. At the same time, we have synthesized *ε*-*N*-Methyl-*ε*-*N*-Methacryllysine (MemeaK), we are screening the synthetase of this compound. In this study, we focused on Kmea modifications. Without above mentioned tools, it is hard to investigate Kmemea modification. In the future, we will carry out comprehensive Kmemea studies based on pan-specific Kmemea antibodies and genetically encoded MemeaK.

How is the protein Kmea-ylated In the first place. Are there enzymes, i.e. acyltransferases, or is it all non-enzymatically catalyzed? Is there methacryl-CoA in cells? If yes, how is it generated, in which concentrations is it present and in which organelles/subcellular localization do you find it?

Response: We thank the reviewer for his/her comments. Previously, it was reported that histones were methacrylated in nucleus (Delaney K. et al. Cell Discovery 2021). Recently, we found that mitochondria proteins were also mechacrylated, we have submitted the paper to [redacted] (Li Y. et al. [redacted], under review). Methacrylation is enzymatically catalyzed, and HAT1 is the methacryltransferase. Metharylyl-CoA is the intermediate metabolite in the mitochondrial catabolism of valine (Figure R7), which is present in mitochondria (Peters H. et al. Molecular Genetics and Metabolism, 2015).

[Redacted figure]

Figure R7(Cited from Peters H. et al. Molecular Genetics and Metabolism, 2015). Valine catabolic pathway showing the formation of metabolites in HIBCH deficiency Enzymes are numbered: 1 = 3-hydroxyisobutyryl-CoA hydrolase; 2 = propionyl-CoA carboxylase; 3 = (R)-methylmalonyl-CoA mutase; 4 = short-chain enoyl-CoA hydratase (ECHS1 gene); 5 = isobutyryl-CoA dehydrogenase. Acryloyl-CoA as the possible metabolic origin of 2,3-dihydroxy-2-methylbutyric was indirectly inferred.

What is the stoichiometry of Kmea at K125 and/or K131 in CypA? Is that sufficient to generate a physiologically important effect? This should be discussed.

Response: The stoichiometry of Kmea at CypA K125 and CypA K131 is low, but it did affect protein interactome of CypA. We have performed functional study about Kmea and revealed that genetically encoded Kmea could recapitulate the mitochondrial morphology defects observed in cells lacking HIBCH or ECHS1 (Li Y. et al., under review).

2. The figure legends do not contain sufficient information to understand what is shown. Several figures in the main text and the Supplementary Information file are not corresponding to the figures and panels within the figures. Please check this carefully. The lenges must contain all essential information to be able to understand what is shown.

Response: We thank the reviewer for his/her comments. We have corrected them.

3. How was Kmea-ylation identified in CypA? Was that by MS on the endogenous protein level? Pelase explain this more precisely.

Response: We thank the reviewer for his/her comments. In this study, we identified Kmea of CypA by strep-tagged IP and tandem mass spectrometry identification. In another study (Li Y. et al., under review), we identified endogenous Kmea of CypA by pan-specific Kmea antibody enrichment and tandem mass spectrometry identification.

4. How is Kmemea generated from Kmea? Is there a methyltransferase? If it is no known, please discuss this.

Response: We thank the reviewer for his/her comments. In this study, we focused on Kmea modification, and we don't know which enzyme is responsible for methylation of Kmea on CypA. We are developing pan-specific Kmemea antibody and ncAA MemeaK. These new tools will be helpful for mapping methylase of CypA Kmea.

5. CypA was previously shown to be acetylated and the impact of acetylation was reported. Does Kmea or Kmemea also affect CsA binding? If it affects CsA binding it can be speculated that it also affects substrate binding. How is the catalytic activity affected by Kmea/Kmemea at K125 or K131?

Response: We thank the reviewer for his/her comments. To investigate the effect of CypA methacrylation on CsA binding, we compared the affinity of CsA to methacrylated and unmethacrylated CypA by MicroScale Thermophoresis(MST). We measured a CypA–CsA dissociation constant (350 nM). Measurements of methacrylated CypA binding to CsA, however, showed that methacrylation reduced the affinity of CypA for CsA by 8-fold (Kd 3.23 μM, Figure R8). We didn't focus on catalytic activity of CypA in this study.

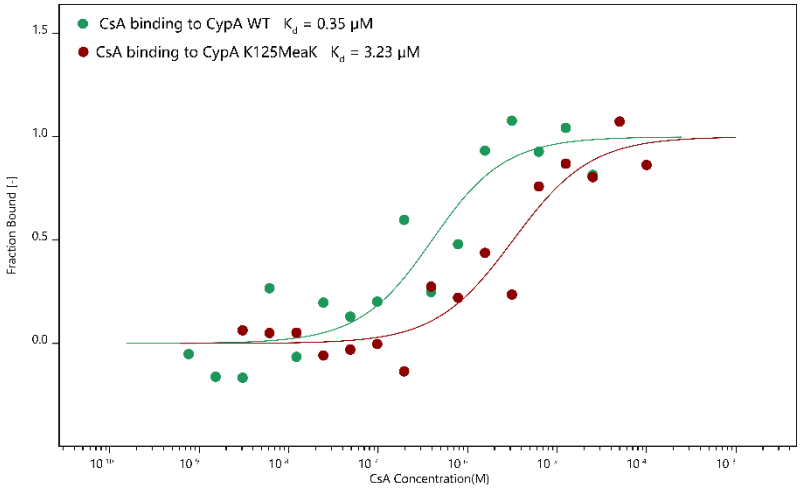

Figure R8 (Supplementary Figure 11). C-terminal of CypA WT and CypA K125MeaK were fused with GFP. Binding affinity of CSA and GFP fused proteins were measure by MST binding affinity analysis. Data are presented as mean ± SD. Kd values are shown. CypA WT exhibited stronger binding to CsA than K125MeaK mutations. Technical replicates of the MST measurements were performed (n = 3).

6. Which antibody did the authors use to detect Kmea? It is specific for Kmea or does it also detect Kcr? Please provide data showing the antibody is specific.
Response: We thank the reviewer for his/her comments. The pan specific anti-Kmea antibody was purchased from PTM BioLab, Inc. (Catalog No. PTM-1501). The pan specific anti-Kmea antibody has 20-fold more specific binding activity than crotonylated peptides (Delaney K. et al. Cell Discovery, 2021). The results of the above experiments were repeated with our purchased antibody (Figure R1).

7. The authors describe they used the crosslinkers BVSB and PDES to crosslink CypA. How was that experimentally done? Didi they overexpress CypA? Please add this information.
Response: We performed whole proteome cross-linking in live cells with BVSB and PDES followed by His-tag thioredoxin pull-down experiment, endogenous CypA was identified to cross-link with thioredoxin in thioredoxin pull-down samples (Wu T. et al. Redox Biology, 2023). We have added this part to the manuscript.

8. The peptides the authos show to be crosslinked in Fig. 1a suggest that there is a simple disulfide bond formed. Can the authors clarify this.
Response: This peptide is BVSB mediated cross-linked peptide, but not disulfide bond linked peptide.

9. Fig. 1 b shows the table of identified CypA interaction partners. Can the authors explain why they decided to study thioredoxin? There were only two unique peptides identified, which is much less as observed for SPTSH and NCOR1.
Response: Previously, we identified TXN1 binding protein CypA in TXN1 His-tag pull-down. To validate this protein-protein interaction. we performed CypA strep IP and TXN1 was

identified. Molecular weight of TXN1 is only 11kD and most of trypsin digested peptides on TXN1 are too long or two short which are not suitable for mass spectrometry analysis. Therefore, limited peptides of TXN1 were identified in CypA IP. Molecular weight of SPT5H and NCOR1 are more than 100kD proteins and they have longer sequences.

10. The authors suggest interaction of CypA and thioredoxin, Kmea-ylation affecting the affinity of the interaction. How did they conclude this? A first indication would be if they analyzed the interaction in vitro by performing a protein-protein interaction study by SPR, ITC, MST or similar.

Response: We apologize for the confusion. We changed this description 'has stronger binding affinity' to 'has stronger interactions'. More TXN1 was precipitated in CypA K125MeaK IP than CypA K125A and CypA WT IP, suggesting that CypA K125MeaK has relative stronger interactions with TXN1 than CypA K125A and CypA WT.

11. Fig. 1e. It is not mentioned which antibody is used. How often was the experiment performed. Can the quantify the blot?

Response: The pan specific anti-Kmea antibody was purchased from PTM BioLab, Inc. (Catalog No. PTM-1501). This antibody was published in this paper (Delaney K. et al. Cell Discovery, 2021). We repeated the experiment and quantified the blots (Figure R9).

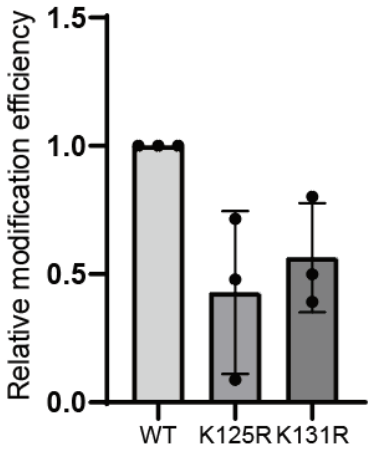

Figure R9 (Supplementary Figure 1c). Western blot statistical analysis of methacrylation levels in CypA and its K125R/K131R mutant (n = 3). Data are normalized by CypA intensity, and methacrylation levels were shown as ratios to WT. Data are presented as mean ± SD.

12. The authors describe identification of Kmea by LC-MS/MS due to formation of an cyclic immonium ion similar as was described for lactylation. Did the authors detect Kmea at all in the peptides or was the detection purely done by generation of the cyclic immonium ion? Did the authors also detect the linear immonium ion? I think for lactylation it was detected.

Response: We applied high-resolution mass spectrometry to identify Kmea peptides. Identification of Kmea was supported by multiple continuous b or y ions, not only the cyclic immonium. We observed linear immonium ions on part of tandem mass spectra, not all of the mass spectra, so we haven't shown it here.

13. Fig. 2b. How was the Y349 selected as a residue being critical for the improved PylRS variant to charge the tRNA with methacryllysine? Can the authors also give numbers how much more efficient the Y349F variant was compared to PylRS? The figure is almost not readable. Please prepare a figure that shows clearly what you want to show.
Response: *Mb*PylRS Y349F (as *Mm*PylRS Y384F) has higher in vivo amber suppression activities than PylRS WT. One potential explanation is that Y384F mutation may affect the opening/closing of the amino acid binding tunnel and regulate $tRNA^{Pyl}$ accessing the enzyme-bound pyrrolysyladenylate (Yanagisawa T. et al. Chemistry & Biology, 2008). For MeaK incorporation, activity of *Mb*PylRS Y349F is slightly higher than *Mb*PylRS WT (Figure R10).

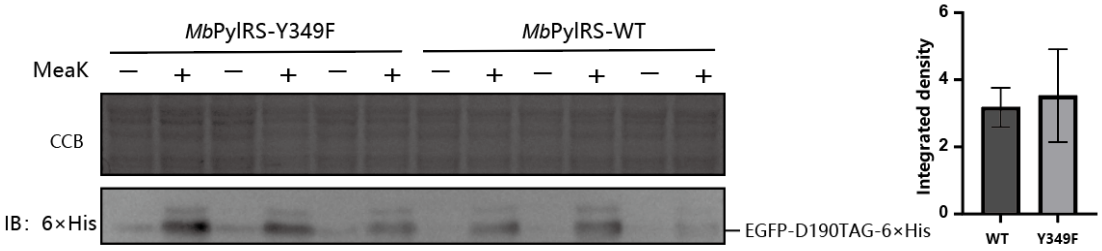

Figure R10. Comparison of MeaK incorporation efficiency between *Mb*PylRS Y349F and *Mb*PylRS WT. Western blot shows MeaK incorporated EGFP by *Mb*PylRS Y349F or *Mb*PylRS WT. The loading control was *E. coli* whole cell lysates analyzed by Coomassie brilliant blue (CBB) stained SDS-PAGE gel. The intensity of EGFP was normalized by loading control. Three biological replicates were performed.

14. For the recombinant expression of MBP-Z they used a different PylRS variant compared to the cellular studies later. Can the authors explain why that was necessary?
Response: Generally, ncAA incorporation efficiency in mammalian cell is less than in *E. coli*, so we used the chimeric PylRS to improve ncAA incorporation efficiency in mammalian cells (Bryson D. et al. Nature Chemical Biology, 2017).

15. How specific are the improved PylRS variants for Kmea charging the cognate tRNA and the subsequent incorporation? Do the enzymes not incorporate any other amino acid. PylRS is rather unspecific and it results in incorporation of other amino acids as well.
Response: *Mb*PylRS Y349F and *Mm*PylRS Y384F can recognize multiple ncAAs. When we culture the cells, we just treat cell with MeaK. So, it won't incorporate other ncAAs. When we don't feed cells with MeaK in negative control, *Mb*PylRS Y349F will take natural amino acid. If the cells were treated with MeaK, incorporation of natural amino aicd will be dramatically suppressed.

16. Microscopy images: almost all microscopy images are not visible and it is a really hard task to see what one should see. Please provide improved images.
Response: For the microscopy images, we are not aimed to show single cell. We want to show the fluorescence of cell populations.

17. Can you provide a ESI-MS molecular weight determination of the whole MBP-Z protein?

That will show if the protein is quantitatively Kmea-ylated. For EGFP-Y15Kmea (sometimes the authors write Kmea, sometimes MeaK, please be consistent) the authors also detect EGFP-Y15K protein (Fig. 1h). Can the authors explain how this was occurring? Does the improved PylRS-variant also charge PylT with K?

Response: The mass spectrum of integrated MBP-Z is shown in Figure R11. We apologize for the confusion. MeaK is the ncAA of ε-N-Methacryllysine, while Kmea is the type of protein modification. The EGFP Y151K may be due to demethacrylation of EGFP Y151MeaK by endogenous deacetylases.

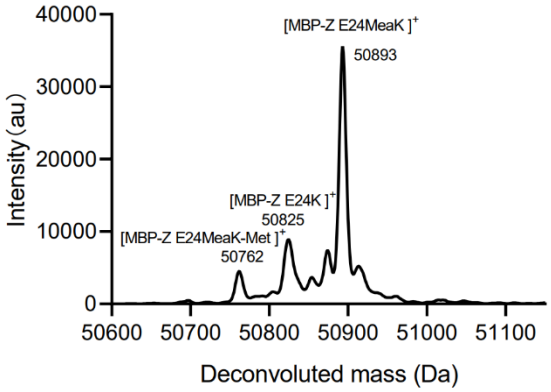

Figure R11 (Figure 2d). Intact protein mass analysis of *E. coli* cells expressed MBP-Z E24MeaK.

18. For some peptides, such as in Fig. 1i, you write “U” in the fragmentation spectrum. Does this mean there is a Kmea and in the peptides, the authors write a “K”, that there is a K and the Kmea can only be identified by the cyclic immonium ion? Please clarify. In some figures, such as Fig. 3c, there is no cyclic immonium ion shown. Why is there no cyclic immonium ion?

Response: ‘U’ represents ncAA MeaK that was incorporated into this site. Red ‘K’ represents the methacrylated lysine which was modified by enzymes in cells, no ncAA incorporation. The methacrylated peptides were identified based on high mass accuracy precursor, multiple b ions and y ions and the cyclic immonium ion. When we discuss about the cyclic immonium ion, we only labeled the cyclic immonium ion in the mass spectrum. For all the Kmea peptides in this manuscript, they do have cyclic immonium ion. However, we didn’t have to label cyclic immonium ion in all mass spectrum to avoid.

19. The authors consistently write “upregulated”/”downregulated” for the MS-IP data. This is wrong. The authors do not perform gene expression studies. It only means that they either identified proteins interaction stronger with one variant or better with another variant. In that context it does not become clear why the authors only focused on the improved interactions, while they did not say anything about proteins, which bind stronger to Cyp WT compares to the Kmea-ylated proteins.

Response: To avoid the confusion, we have changed ‘upregulated proteins’ and ‘downregulated proteins’ to ‘upregulated hits’ and ‘downregulated hits’, as shown in the manuscript. The expression of CypA K125MeaK is less than CypA WT and CypA K125A.

Under this situation, the upregulated hits are really upregulated. For the downregulated hits, we can not differentiate that they are real downregulated hits or caused by low expression level of CypA K125MeaK. So, we focused on the upregulated hits.

20. It would be interesting to discuss how a Kmea-ylation would differently regulate CypA function compared to acetylation or other acylations. Can the authors discuss this.
Response: We have identified binding proteins of CypA K125CrK and CypA K125MeaK (Figure R4), and discussed the difference of their binding partners.

21. Fig. 3d. Information is missing where the nanobody comes from (also for the image in the Supplementary information file).
Response: Nanobody was purchased from the Strep beads (Smart-Lifesciences Catalog Number: SA053100). We have added this information to the method section.

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22. In Fig. 3b and Supp. Fig. 6 the authors describe the observation that in selected tumor cells acetylation at K125 and K131 is stronger or reduced compared to the non-acytlated protein. The pattern is different for K131 and K125. Can the authors provide a mechanism that underlies these differences. The authors do not give a real reference for the data, they derived the hypothesis from.
Response: We thank the reviewer for his/her comments. We downloaded six proteome-wide acetylation datasets from CPTAC and analyzed acetylation site of CypA from these datasets. Acylation of CypA was reported to be essential for the proliferation of pulmonary arterial endothelial cells (Mao M. et al. J Hypertens, 2017). Acylation of CypA also stimulates increased in inflammation, and oxidative stress in human pulmonary microvascular endothelial cells (Xue C. et al. Arterioscler Thromb Vasc Biol, 2017). And both Fig3b and FigS6 data also showed that acetylation at K125 and K131 was enhanced in lung adenocarcinoma.

23. Fig. S5. Which antibody was used? If you stain with anti-Kmea antibody and with anti-Cyp-antibody the authors would habe a loading control.
Response: We thank the reviewer for his/her comments. We repeated the experiment, labeled the antibodies and added loading control to Figure R12 (Figure S5).

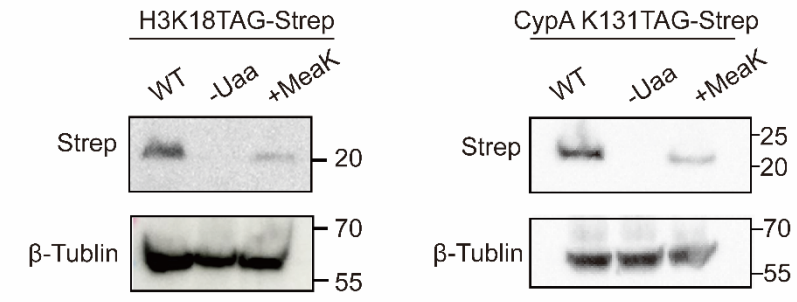

Figure R12 (Figure S5). Western blot of cell lysates from HEK293T cells expressing CypA WT and K131TAG; H3 WT and K18TAG.

24. In the MS-data in Fig. S7 and S8 the level is lower for Kmea-ylated protein compared to CypA K125A and WT. Is the data corrected for different protein abundances? In fif. S8 an SDS-PAGE is provided, in Fig. S7 not, is there a reason? In Fig. S8b the nanobody band is not labelled. Legend of Fig. S8 does not correspond to the figure.
Response: Our MS-data were processed using quantile normalisation. The expression level of MeaK incorporated CypA is lower than CypA K125A and WT. The SDS-PAGE gel related to Fig. S7 is Fig. 3d. We have labeled 'nanobody' on Fig. S8b and corrected figure legend of Fig. S8.

25. Fig. S9, S10, S11 is only one single sentence. This is not sufficient information to understand what is shown. The cell images are almost invisible.
Response: We have rewritten the figure legend of Fig. S9-11. In the fluorescent image, we want to show the cell population, but not single cell.

26. Fig. S12. The SIRT2 blot suggests unspecific binding.
Response: We agree with the reviewer that SIRT2 bind unspecifically with the beads. However, the binding of SIRT2 to CypA conjugated beads is stronger than the beads, suggesting that SIRT2 also bind with CypA.

27. In Fig. 5a the authors write "NAM" to indicate an inhibition of deacetylases, later they only use butyrate. Importantly, nicotinamide is a non-competitive inhibitor only for sirtuins and butryrate is a quite low-potency inhibitor only for classical deacetylases. The authors suggest CypA being deacetylated by both enzyme classes. Why did they not include NAM and butyrate. As a suggestion, SAHA and TSA are also pan-HDAC inhibitors, however, with a much higher potency in the nanomolar range compared to butyrate.
Response: We apologize for the mistake. We have corrected it in Fig. 5a. We used nicotinamide and butryrate as suggested in this paper (Delaney K. et al. Cell Discovery, 2021). We thank the reviewer for his/her suggestions about SAHA and TSA. However, the study using nicotinamide and butryrate is already conclusive. In the future, we will try the pan-HDAC inhibitors SAHA and TSA in our acylation researches.

28. It is not clear why the authors used the technique with the self-cleavable peptide. This needs a better description.
Response: We used mCherry proteins as an internal control. The self-cleavable peptide allows mCherry and EGFP to separate and fold independently after translation.

29. Fig. 5d. The assay format for HDAC2 is different from HDAC1 and HDAC3. It cannot be concluded if HDAC2 is capable to act as deacetylase. The experiment must be repeated.
Response: We thank the reviewer for pointing this out. We have repeated the experiment. We believe now it is conclusive. (Figure R6b).

30. Fig. 5b, bar graphs: Can the authors explain the labelling of the y-axes. Is that relative EGFP fluorescence?
Response: We analyzed the data of this experiments again and the labelling of the y-axes

represent the normalized signal of EGFP to RFP. We measured the intensity of the EGFP and the RFP signal separately using ImageJ and normalized the EGFP signal with the RFP signal for each group.

31. Fig. 4. The axes lack units.
Response: The y-axis counts the number of cells and the x-axis is the relative intensity of the fluorescent signal of FITC. The format of Fig.4 is same as the published figures in this paper (Huang L. et al. Immunity, 2021).

32. Fig. S2: a loading control is missing. The legend is rudimental.
Response: We thank the reviewer for pointing this out, we have prepared this Figure again and rewritten the figure legend (Figure R13).

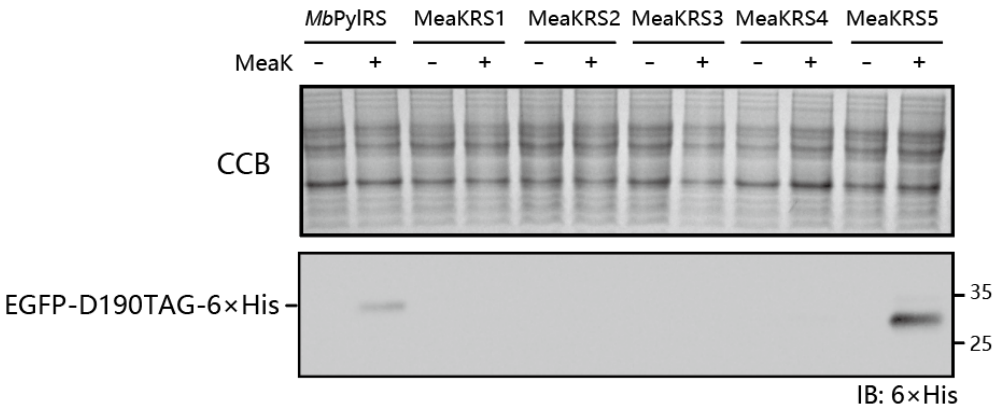

Figure R13 (Figure S2): Western blot of cell lysates from *E. coli* cells co-expressing EGFP D190TAG and *Mb*PylRS mutants. The loading control was shown by Coomassie brilliant blue (CBB) staining SDS-PAGE gel.

33. Fig. S3: another enzyme was generated. Why was that done?
Response: Because the chPylRS Y384F has higher incorporation efficiency in mammalian cells (Bryson D. et al. Nature Chemical Biology, 2017).

34. Several typos are in the manuscript and the writing is not correct (Fig. S9: “Incorporate MEaK…”; better: “Incorporation of….”; “western” with capital letter, i.e. “Western”;
Response: We apologize for this mistake. We have corrected it.

35. Line 156: “Stronger binding affinity was detected”. How was that determined?
Response: We have changed the description of ‘Stronger binding’ to ‘Stronger interaction’.

36. Avoid using abbreviations such as “weren’t” in line 185.
Response: We apologize for this mistake. We have corrected it.

37. The authors present a model according to which Kmea-ylatio of CypA affects ROS production by interfering with CsA binding. Did the authors experimentally measure if

Kmea-ylation of CypA affects CsA binding?

We thank the reviewer for his/her comments. We have already answered this question in an earlier comment. As demonstrated by MST, methacrylation of CypA affects CsA binding (Figure R8).

38. Wording, Line 87,88: "…..of cells and reduce its target proteins wih conserved CXXC motif". It is of course not the motif itself reducing the target proteins. Please rewrite this.

Response: We have changed this sentence to 'cells and reduce its target proteins to protect cell from detrimental effects of ROS'.

39. Line 138: "…but it was mutated to methionine….". What do the authors want to say here? There is a different residue at this position due to the selection pressure during evolution. Can the authors say how the enzyme was phylogenetically developed? Otherwise they cannot make this statement.

Response: We thank the reviewer for his/her comments. The CypA K131 position in the manuscript has mutations to other amino acids in some species, in order to show that K131 is relatively not an evolutionarily conserved amino acid. To illustrate how CypA evolved, we selected protein sequences from more species for comparison and constructed an phylogenetic tree (Figure R14). The results show that *Felis catus* and *Oryctolagus cuniculus*, which produce mutations into Arg and Met, and *Homo sapiens* have similar evolutionary distances although they belong to different nodes downstream. And the frequency of both mutations appears extremely low, suggesting that the mutation is not evolutionarily significant. Notably Glu was instead the higher rated mutation, which was the exact opposite negatively charged amino acid to Lys, suggesting that Lys at position 131 may not be a key amino acid present in both function and folding.

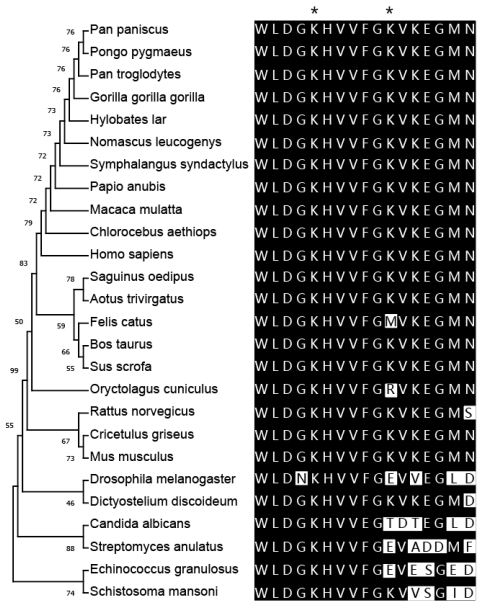

Figure R14. Phylogenetic tree and alignment and conservation of CypA sequences flanking the K125 and K131 site (indicated by an asterisk). The phylogenetic tree was constructed using the Neighbor-Joining method with Bootstrap as 1000.

40. What are “single-chain construct of CypA” (line 178), please explain.
Response: Single-chain means that we constructed the gene sequences of both CypA and mCherry proteins in the same plasmid and co-expressed them in one ORF.

41. Please carefully check if all necessary reference.
Response: We have carefully checked all of the references.

Reviewer #3 (Black: reviewer comments, Blue: our response)

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

Reviewer #4: (Black: reviewer comments, Blue: our response)

The paper entitled “Genetically encoding ε-N-methacryllysine into proteins in live cells” revealed a novel post-translational modification Kmea on a non-histone protein CypA. Besides, the authors developed a general genetic code expansion approach to incorporate an unnatural amino acid (Uaa) ε-N-Methacryllysine (MeaK) into target proteins and identified interacting proteins of methacrylated CypA using affinity-purification MS, by which they found that Kmea at CypA site 125 regulated cellular redox homeostasis and HDAC1 is the regulator of Kmea on CypA. Overall, this paper is written logically. The finding is interesting and meaningful. The conclusions can be supported by their data. This paper can be further improved by addressing some concerns.

1. The authors should clarify their rationale to discuss thioredoxin at the beginning of their story because no background regarding the relationship between Kmea and thioredoxin or redox process was introduced in the introduction.
Response: We thank the reviewer for his/her comments. We have rewritten the beginning of results section.

2. Is CypA the only protein undergoes Kmea modification? If so, the authors should provide an explanation to this unusual finding. IF not, they should include more kmea proteins and explain why they choose to focus on CypA.
Response: We thank the reviewer for his/her comments. We have identified more than 400 Kmea sites in mammalian cells and included these data in another manuscript. This manuscript focused on neuronal disease is under review in [redacted] (Li Y. et al. under review). For this manuscript, we focused on methodology development and chose to study CypA.

3. The authors identified HDAC1 as the eraser of CypA Kmea, which is convincing. How about the writers and readers? Moreover, HDAC1-3 has significant functional similarities and all of them can interact with CypA K125MeaK, why only HDAC1 can regulate CypA Kmea but HDAC2-3 cannot? Please add a few lines to discuss this.

Response: We thank the reviewer for his/her comments. It is challenging to identify readers, writers and erasers of methacrylated CypA with the non-crosslinkable ncAA, MeaK, because the interaction of these proteins with methacrylated CypA are likely weak. We have synthesized photo-crosslinking MeaK, and we are screening the synthetase of this ncAA. The photo-crosslinkable MeaK can site-specifically and covalently capture readers, writers and erasers of CypA Kmea and increase identification sensitivity of binding proteins on methacrylated CypA. In the future, we will apply the photo-crosslinkable MeaK to comprehensively identify readers, writers and erasers of methacrylated CypA and meth-metharylated CypA. CypA has 9 published acetylation sites and 9 crotonylation sites, HDAC 2-3 may involve in de-acylation of other acylation sites.

4. In this paper, the authors used DCFH-DA method to determine ROS level. Although DCFH-DA is a universe method, however, this is not specific to detect hydrogen peroxide, superoxide anion or hydroxyl radicals. Please perform more specific assays to detect cellular ROS.

Response: We thank the reviewer for his/her comments. The DCFH-DA assay is indeed a universe method that we use to detect the degree of cellular oxidative stress associated with CypA. We applied Dihydroethidium (DHE) to detect superoxide anion. Methacrylation of CypA at position K125 leads to a slight increase in intracellular superoxide anion levels (Figure R15). However, the role of intracellular ROS, which was described in the manuscript as being affected by CsA, did not appear to show an effect on superoxide anion. For the components of several other kinds, unfortunately we have not tested them for the present. Considering that our paper was aimed at the development of the technology, and illustrating the biological significance of it should be followed up in further studies.

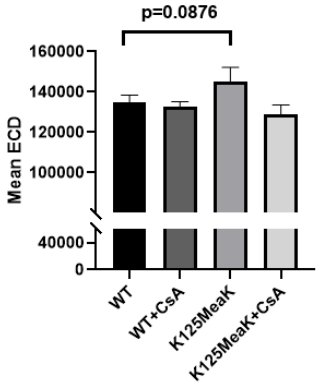

Figure R15. Measurement of degrees of superoxide anion with flow cytometry in WT CypA and CypA K125MeaK expressed HeLa cells in the absence and treated with or without CsA. The same number CypA+ cells were analyzed (n = 3). Data are presented quantitative comparison of superoxide anion levels in different cells. Unpaired 2-tailed Student's t test.

5. The function of CypA Kmea is not clear. As the author used HeLa, a cancer cell line as a cellular model, how does CypA Kmea regulate cancer hallmarks, such as proliferation, metastasis, drug resistance, immune escape, metabolism alteration, or cell death? The authors should at least choose one hallmark to further investigate.
Response: We thank the reviewer for his/her comments. This manuscript is a methodology study. We have performed systematic functional study of Kmea on neuronal disease and submitted the manuscript to [redacted].

Following is the abstract of that manuscript: A deficiency of the valine metabolic enzyme 3-hydroxyisobutyryl-CoA hydrolase (HIBCH) or short-chain enoyl-CoA hydratase (ECHS1) leads to Leigh syndrome, a severe mitochondrial neurodegenerative disease with no cure. We found that the absence of HIBCH or ECHS1 in cultured cells led to abnormal mitochondrial morphology and defects in respiration. Fly eyes lacking either protein showed age-dependent degeneration, which could be rescued by wild-type but not disease-related mutant forms of proteins. Elevated protein lysine methacrylation (Kmea) was observed in both HIBCH- and ECHS1-deficient cells and fly tissues. Quantitative mass spectrometry analysis indicated that many proteins were ectopically modified by Kmea in the HIBCH- or ECHS1-deficient cells. Mimicking Kmea in proteins such as CH60, FKBP4, BIP, or DHRS2 using the genetic code expansion technique could recapitulate the mitochondrial morphology defects observed in cells lacking HIBCH or ECHS1. A reduction in Kmea modification by sirtuin expression or the addition of N-acetyl-L-cysteine (NAC) partially rescued mitochondrial defects in HIBCH- or ECHS1-knockout cells. Treatment of young flies with NAC dramatically suppressed the eye degeneration caused by the loss of Hibch or Echs1. Fibroblasts from human patients with HIBCH or ECHS1 mutations showed mitochondrial defects and elevated Kmea modifications. Administration of NAC to patient fibroblasts reduced Kmea modifications and significantly reversed mitochondrial defects. In summary, we propose that ectopic Kmea modification mediates the defects observed in HIBCH- or ECHS1-deficient cells, flies, and human patients. Reducing Kmea modification provides a new approach for treating HIBCH- or ECHS1-related Leigh syndrome.

6. Throughout the paper, the conclusions are all based on in vitro evidence, which is not compelling. Please perform in vivo model to strengthen this manuscript. For example, an orthotopic mouse model or a PDX mouse model. They should verity their main findings in an in vivo model, including the CypA Kmea modification, the HDAC1 as the eraser of CypA Kmea. To this end, a HDAC1 knockout mouse model is suitable for elucidate that.
Response: We thank the reviewer for his/her comments. Kmea is close related to Leigh syndrome. We have identified de-methacrylases in model organism of *D. Melanogaster* and performed function studies in above mentioned manuscript (Li Y. et al., under review) .

7. The authors found that Kmea on CypA decreases the antioxidant ability of CypA, which is interesting. What is the mechanism underlying Kmea-mediated functional change of CypA? Please perform mechanistic experiments or add a few lines to discuss this.
Response: We thank the reviewer for his/her comments. Peroxiredoxins are members of

peroxidases which can reduce peroxides. CypA was reported to bind with perodiredoxin-6 (PRDX6) and enhance antioxidant activity of PRDX1-6 (Lee S. et al. The Journal of Biological Chemistry, 2001). In our CypA IP experiments, the intensity of PRDX4 in CypA K125MeaK is 3.5-fold lower than that in CypA K125A IP (without normalizing intensity of PRDX4). This data suggests that Kmea of CypA may perturb interaction between CypA and PRDX4 and suppress antioxidant activity of CypA.

8. The authors have to add some clinic relevance to this manuscript. For example, does CypA Kmea exhibit different level between normal and cancerous tissues? Does CypA Kmea hold prognostic value in cancer patients? Is CypA Kmea site (125) frequently mutated in clinic sample?

Response: We thank the reviewer for his/her comments. We performed acetylation analysis in Figure 3b, because Kmea of protein was discovered in 2021 and no Kmea clinical data has been published. We performed Kmea analysis on tissue samples of lung cancer, but the pan-specific Kmea antibody is not suitable for immunohistochemical analysis (Figure R16), Also, the CypA K125kmea antibody is not available. We did not find the clinic sample mutation in the cBioPortal (cBio Cancer Genomics Portal) database for the CypA at 125.

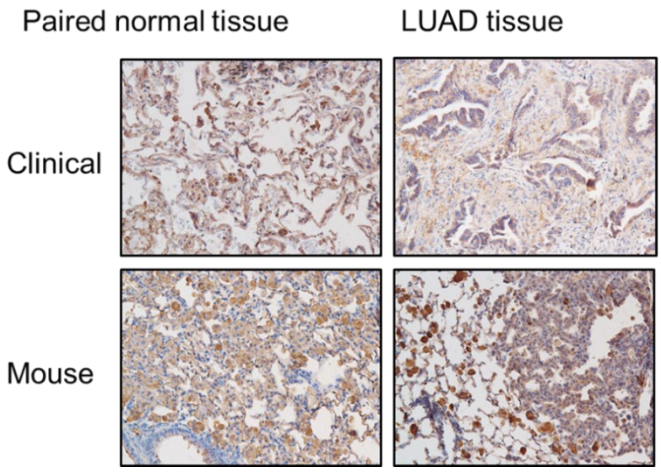

Figure R16. Immunohistochemical staining of Kmea modification in lung adenocarcinoma (LUAD) tissues and paired healthy tissues.

9. In this paper, the authors found that CypA Kmea can be further methylated (Kacme), which is interesting. They have to clarify the functional differences between Kmea and Kacme on CypA. Given that Kmea significantly regulates the antioxidant capability of CypA as the authors found, what is the impact of Kacme on CypA antioxidant function?

Response: We thank the reviewer for his/her comments. We are in the process of screening the pan-specific Kmemea antibody with peptide library. Without this antibody, it is hard to identify endogenous Kmemea site and perform functional study. At the same time, we have synthesized MemeaK compound and are performing screening the tRNA synthetase that can recognize MemeaK. We hope to address these questions in the near future.

Reviewer #1 (Black: reviewer comments, Blue: our response)
The revision has significantly improved the quality of the manuscript. The authors should address the following remaining issues prior to publication:
- The tRNA used should be stated alongside the Synthase for each Experiment.
Response: We thank the reviewer for pointing this out, we stated the tRNA for the plasmids used in the manuscript and method.

- chPylRS reference still refers to chPheRS paper, which was not used according to the authors. The reference should be corrected to Bryson D.
et al. Nature Chemical Biology, 2017 as stated in the review.
Response: We apologize for this mistake; we have corrected it.

- Spelling of Cyclosporin(e) is inconsistent.
Response: We thank the reviewer for his/her comment, we have corrected it.

- Conclusions made to PRDX1-6 are poorly supported.
Response: We thank the reviewer for his/her comments. In this study, we mainly developed the genetic code expansion technology and just discussed the data of PRDX1-6. In the future, we will focus the functions of PRDX1-6 in cancer cells.

- General language editing is strongly advised.
Response: We thank the reviewer for his/her comments, we have edited the language of this manuscript.

Reviewer #2 (Black: reviewer comments, Purple: reviewer response, Blue: our response)
Review to the revised manuscript entitled “Genetically encoding e-N-methacryllysine into proteinsin live cells” submitted by Zhu and co-workers Zhu et al. submitted a revised manuscript describing the site-specific incorporation of methacryl-lysine (Kmea) into proteins using a synthetically evolved aminoacyl-tRNA-synthetase/tRNACUA pair based on the PylRS/PylT from Methanosarcina barkeri. Overall, the manuscript improved but there are still some open issues. An important issue is the authors rplying to several of m y points of criticism by citing the manuscript which is currently under review in [redacted]. From my point of view this is not a valid answer. The open questions should be addresse d concisely and if there is additional data which is part of another manuscript which is und er review in a different journal, the authors should show the data here as well. The editor should make the decision if this kind of answer is ok for Nature Communication or not. M oreover, my earlier comments were sometimes not completely answered and the authors only answered some aspects of my comments. Please go through my earlier comments a nd provide answers to all my points of criticisms. Here are the open issues, written below the responses of the authors:

1. The text is written very superficial in almost all sections. The discussion does not contain any information on regulation of Kmea/Kmemea in cells. It Kmemea also removed by classical deacetylases or sirtuins?

Response: We thank the reviewer for his/her comments. We have shown that HDAC1 is responsible for removal of Kmea. For Kmemea, we are generating pan-specific Kmemea antibodies with peptide library, but it is challenging to get high specificity Kmemea antibodies. At the same time, we have synthesized ε-N-Methyl-ε-N-Methacryllysine (MemeaK), we are screening the synthetase of this compound. In this study, we focused on Kmea modifications. Without above mentioned tools, it is hard to investigate Kmemea modification. In the future, we will carry out comprehensive Kmemea studies based on pan-specific Kmemea antibodies and genetically encoded MemeaK.

Response:
Ok, just be sure that you give more details.

2. How is the protein Kmea-ylated In the first place. Are there enzymes, i.e. acyltransferases, or is it all non-enzymatically catalyzed? Is there methacryl-CoA in cells? If yes, how is it generated, in which concentrations is it present and in which organelles/subcellular localization do you find it?
Response: We thank the reviewer for his/her comments. Previously, it was reported that histones were methacrylated in nucleus (Delaney K. et al. Cell Discovery 2021). Recently , we found that mitochondria proteins were also mechacrylated, we have submitted th e paper to [redacted] (Li Y. et al., under review). Methacrylation is enzymatically cat alyzed, and HAT1 is the methacryltransferase. Metharylyl-CoA is the intermediate me tabolite in the mitochondrial catabolism of valine (Figure R7), which is present in mitoc hondria (Peters H. et al. Molecular Genetics and Metabolism, 2015). Figure R7(Cited fro m Peters H. et al. Molecular Genetics and Metabolism, 2015). Valine catabolic pathway showing the formation of metabolites in HIBCH deficiency Enzymes are numbere d: 1 = 3-hydroxyisobutyryl-CoA hydrolase; 2 = propionyl-CoA carboxylase; 3 = (R)-methyl malonyl-CoA mutase; 4 = short-chain enoyl-CoA hydratase (ECHS1 gene); 5 = isobutyryl -CoA dehydrogenase. Acryloyl-CoA as the possible metabolic origin of 2,3-dihydrox y-2-methylbutyric was indirectly inferred.

Response:
HAT1 is an ac(et)yltransferase predominantly present in the cytosol (see many publications on HAT1 by the lab of John Denu and others). According to the response of the authors methacrylyl-CoA is present in the mitochondrial matrix. How can the methacrylation be enzymatically regulated in the mitochondria. Is there evidence that HAT1 enters the mitochondrial matrix? Recently, MOF was shown by the Akthar lab to localize to the mitochondrial matrix but no other lysine acyltransferase is present in the mitochondrial matrix. Can MOF lysine mathacrylate proteins in the mitochondria? Along this line, if methacrylyl-CoA is generated in the mitochondrial matrix how is a cytosolic pool of methacrylyl-CoA established? Please discuss this discrepancy in the paper. No answer is given to the concentration of methacrylyl-CoA in cells (cytosol, nucleus, mitochondrial matrix). What is the KM-value of HAT1 for methacrylyl-CoA? Might it not possible that methacrylation in the mitochondria (and in other cellular compartments) occurs non-enzymatically as well as reported for most other lysine acylations? Please not only answer

it here but also discuss it in the paper.

Response: We thank the reviewer for his/her comments. In this study we haven't identified any acyltransferases, and genetically encoded MeaK is not suitable for capturing the acyltransferases. So, we haven't discussed about acyltransferases in this manuscript. HAT1 is responsible for methacrylation of Histone but not mitochondria proteins (Delaney K. et al. Cell Discovery 2021). Genetically encoded MeaK depends on concentration of MeaK but not endogenous methacrylyl-CoA, we don't know the concentration of methacrylyl-CoA in cells. The concentration of endogenous methacrylyl-CoA is not closely related to our study. The technology developed in this study is not good for mapping acyltransferases, so we haven't discussed about acyltransferases in this manuscript.

[Figure redacted]

Figure R1. Kmea modification of DHRS2 K206 site led to mitochondrial defects. (a) Genetic codon expansion techniques were used to incorporate methacryllysine site-specifically into K206 in DHRS2. Cells were transfected with plasmids encoding a pair of $tRNA^{pyl}_{CUA}$/chPylRS Y384F together with a plasmid encoding wild-type DHRS2 and treated with MeaK (WT + MeaK, left panel). These cells served as a control. Cells were transfected with plasmids encoding a pair of orthogonal aaRS and tRNA together with a plasmid in which the DHRS2 K206 codon was mutated to TAG. No MeaK was added (TAG–MeaK, middle panel). These cells served as a second control. Cells were transfected with plasmids encoding a pair of orthogonal aaRS and tRNA together with a plasmid in which the DHRS2 K206 codon was mutated to TAG. These cells were treated with MeaK (TAGmu + MeaK, right panel). Under these conditions, MeaK could be incorporated at residue 206. Representative confocal images showing alterations in mitochondrial morphology (green) after overexpressing DHRS2 with MeaK incorporated at residue 206 (red). Scale bar: 10 μm. (b) The ratio of the transfected cells with defective mitochondria in the experiments described in (a) was quantified. The expression of wild-type DHRS2 did not change mitochondrial morphology. Mitochondria were fragmented in DHRS2 K206mea-expressing cells. n=40.

3. What is the stoichiometry of Kmea at K125 and/or K131 in CypA? Is that sufficient to generate a physiologically important effect? This should be discussed.

Response: The stoichiometry of Kmea at CypA K125 and CypA K131 is low, but it did affect protein interactome of CypA. We have performed functional study about Kmea and revealed that genetically encoded Kmea could recapitulate the mitochondrial morphology

defects observed in cells lacking HIBCH or ECHS1 (Li Y. et al., under review).
Response:
How can a low CypA modification stoichiometry affect the CypA interactome so drasticall y? Can the authors provide explanations. Does the modification result in a gain-of-functio n effect, i.e. it generates novel functions so that a lower stoichiometry would be sufficient ? The authors could discuss gain-of-function effects and loss-of-function effects. For a los s-of-functions higher stoichiometry would be essential but not for a gain-of-function. Or co uld the modification affect other processes apart from directly modulating protein-pro tein interactions, such as acting at the transcriptional level, etc. Again, the authors state t he manuscript under review in [redacted]. If they have important data for this manuscript t hey should present it here as well. Importantly, genetically encoding the modification results in much higher stoichiometries compared to the stoichiometry present at endogenous levels. This should also be discussed.

Response: The stoichiometry of endogenous Kmea at CypA K125 and CypA K131 is low, but these signals can be amplified by the following signaling pathway. We have not identified the interactome of endogenous methacrylated CypA, we identified the interactome of CypA K125MeaK in this study. The methacrylation level of CypA K125Me aK is high. Mitochondrial fragmentation caused by the genetically encoded DH RS2 K206MeaK is investigated in manuscript which is under review in [redacted]. (Figure R1).

4. How was Kmea-ylation identified in CypA? Was that by MS on the endogenous protein level? Pelase explain this more precisely.

Response: We thank the reviewer for his/her comments. In this study, we identified Kmea of CypA by strep-tagged IP and tandem mass spectrometry identification. In another stud y (Li Y. et al., under review), we identified endogenous Kmea of CypA by pan-specific Km ea antibody enrichment and tandem mass spectrometry identification.

Response:
Again, please show the data here and not just cite the paper in [redacted]. The modification of CypA was found by strep-tag IP as stated by the authors. Please show th e IP here as well and not just in the [redacted] paper. Was it important to cultivate the cell s under specific conditions affecting cellular methacrylyl-CoA levels?

Response: In another study (Li Y. et al., under review), we have performed stable isotop e labeling by amino acids in cell culture (SILAC) quantitative analysis of endogenous Kmea sites in U2OS WT, HIBCH-Knockout or ECHS1-Knockout cells (Figure R2a). Endo genous Kmea of CypA was identified in all of the cell types (Figure R2b).

5. How is Kmemea generated from Kmea? Is there a methyltransferase? If it is no known, please discuss this.

Response: We thank the reviewer for his/her comments. In this study, we focused on Kmea modification, and we don't know which enzyme is responsible for methylation of Kmea on

CypA. We are developing pan-specific Kmemea antibody and ncAA MemeaK. These new tools will be helpful for mapping methylase of CypA Kmea.

Response:
Please discuss it in the manuscript as stated in my comments.

Response: We thank the reviewer for his/her comments, and we have added this part of the discussion in the revised manuscript.

[Redacted]

Figure R2. Quantification of endogenous Kmea site of CypA. (a) A diagram showing the experimental workflow for SILAC and mass spectrometry for quantitatively detecting differential Kmea modification sites in WT control vs HIBCH KO or WT control vs ECHS1 KO cells. (b) Mass spectrometry quantitation results of Kmea site on CypA.

6. CypA was previously shown to be acetylated and the impact of acetylation was reported. Does Kmea or Kmemea also affect CsA binding? If it affects CsA binding it can be speculated that it also affects substrate binding. How is the catalytic activity affected by Kmea/Kmemea at K125 or K131?
Response: We thank the reviewer for his/her comments. To investigate the effect of CypA methacrylation on CsA binding, we compared the affinity of CsA to methacrylated and unmethacrylated CypA by MicroScale Thermophoresis(MST). We measured a CypA–CsA dissociation constant (350 nM). Measurements of methacrylated CypA binding to CsA,

however, showed that methacrylation reduced the affinity of CypA for CsA by 8-fold (Kd 3_._2_3_ _μM, Figure R8). We didn't focus on catalytic activity of CypA in this study.

Response:
It is good that the authors measured the affinities of the modified and unmodified CypA. However, the data seems to be of low quality. Please be sure that you describe exactly how you did the assay. Which fluorescent label was used, which concentrations, which buffers, etc. The data looks very scattered and the signal is really weak. Please provide quality indicators, including controls, known for MST showing the signal you see is real. A control could be a CypA mutant that does not bind CsA anymore. Particularly looking at the weak and scattering data such a control is really important. The figure legend states that the mean+/- SD is shown but this is not the case. I only see the measurement of. Single experiment.

Response: We thank the reviewer for his/her comments. MST Analysis was performed in buffer (50 mM HEPES, 150 mM NaCl and 5% DMSO). Protein was fused with EGFP as a fluorescent signaling. The protein concentration was 100 nM, 5μL of target protein was mixed with 5 μL of 2-fold serially diluted CsA at room temperature, which is described in methods. For quality control, during the MST experiments we ensured that the proteins were unaggregated. The buffers for each dilution group were exactly the same, and the variation in initial fluorescence of all samples in each group was within ±20% before data collection. We have searched the literature and have not found a mutation of CypA can block the binding of CsA. The data are the result of three biological replicates, and we have added error bar for reference (Figure R3).

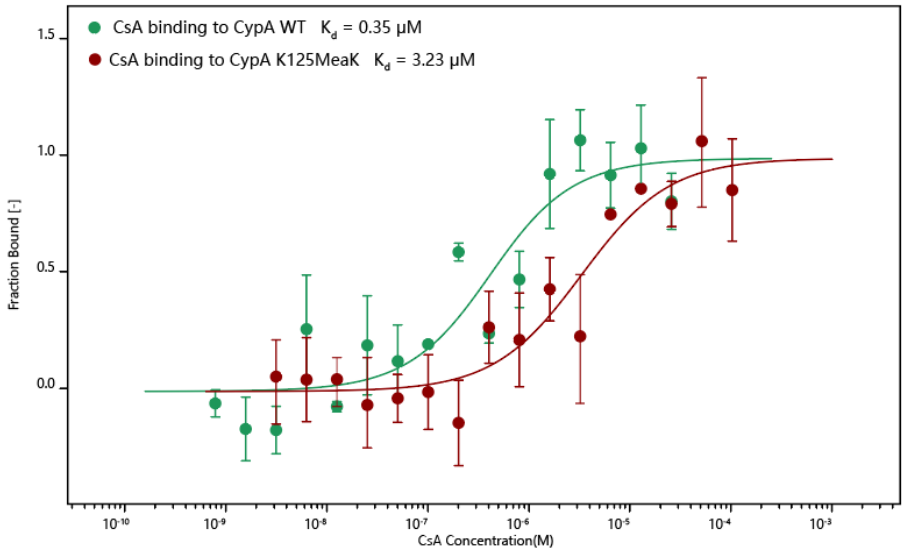

Figure R3. C-terminal of CypA WT and CypA K125MeaK were fused with GFP. Binding affinity of CsA and GFP fused proteins were measure by MST binding affinity analysis. Data are presented as mean ± SD. Kd values are shown. CypA WT exhibited stronger binding to CsA than K125MeaK mutations. Technical replicates of the MST measurements were performed (n = 3).

7. The authors suggest interaction of CypA and thioredoxin, Kmea-ylation affecting the affinity of the interaction. How did they conclude this? A first indication would be if they analyzed the interaction in vitro by performing a protein-protein interaction study by SPR, ITC, MST or similar.

Response: We apologize for the confusion. We changed this description 'has stronger binding affinity' _to 'has stronger interactions'. More TXN1 was precipitated in CypA K125MeaK IP than CypA K125A and CypA WT IP, suggesting that CypA K125MeaK has relative stronger interactions with TXN1 than CypA K125A and CypA WT.

Response:

I do not see what the authors want to say here. What is the point saying "stronger interactions" instead of "stronger binding". Important is that the protein level was similar for the CypA WT and the variants if concluding a higher binding affinity from a stronger signal in the IP immunoblotting.

Response: We thank the reviewer for his/her comments. Mass spectrometry is powerful quantification tool. If more proteins were identified in CypA125 MeaK IP than CypAK125A IP and CypA WT IP (Fig. 3c and Supplementary Fig. 7c), it represents that these proteins have stronger interactions with CypA125 MeaK than CypAK125A and CypA WT.

8. Fig. 1e. It is not mentioned which antibody is used. How often was the experiment performed. Can the quantify the blot?

Response: The pan specific anti-Kmea antibody was purchased from PTM BioLab, Inc. (CatalogNo. PTM-1501). This antibody was published in this paper (Delaney K. et al. Cell Discovery, 2021). We repeated the experiment and quantified the blots (Figure R9).

Response:

The figure shows a lower modification efficiency for the mutants compared to CypA wildtype. Interesting to see would be the double mutant, i.e. K125R and K131R, to see if they are additive. Please also explaion in the manuscript how the experiment was done.

Response: We agree with the reviewer, it is interesting about combination of Kmea on CypA and crosstalk between Kmea and other acylation modifications. In the future, we will apply Top-down or Middle-down proteomics strategies to precisely study the acylation pattern on CypA.

9. Fig. 2b. How was the Y349 selected as a residue being critical for the improved PylRS variant to charge the tRNA with methacryllysine? Can the authors also give numbers how much more efficient the Y349F variant was compared to PylRS? The figure is almost not readable. Please prepare a figure that shows clearly what you want to show.

Response: *Mb*PylRS Y349F (as *Mm*PylRS Y384F) has higher in vivo amber suppression activities than PylRS WT. One potential explanation is that Y384F mutation may affect the opening/closing of the amino acid binding tunnel and regulate tRNAPyl accessing the enzyme-bound pyrrolysyladenylate (Yanagisawa T. et al. Chemistry & Biology, 2008). For

MeaK incorporation, activity of *Mb*PylRS Y349F is slightly higher than *Mb*PylRS WT (Figure R10).

Response:
What do the authors mean with “has higher in vivo amber suppression activities”? Please explain. Is that true for different lysine acylations or incorporation efficiency observed for other unnatural amino acids? In fact the quantification shows no significant difference in incorporation efficiency for PylRS WT and PylRS Y384F. It is known that PylRS does incorporate other amino acids (also natural amino acids) and it is really important to be sure that the protein observed does really contain (at least to the most extend) the lysine methacrylated protein. So, please provide also an immunoblotting with anti-Kmea-antibody for all figures you incorporate Kmea and not just show CCB (Coomassie brilliant blue; typo in the labelling in figure R10!)) staining anti-His6 immunoblots. This shows the generation of full length proteins, i.e. proteins which were not truncated due to the amber stop codon, but not the presence of Kmea.

Response: “Amber suppression activities” should be “Amber codon suppression activities”, it means that the synthetase produced aminoacyl tRNA can suppress amber codon. Yes, there is no significant difference in incorporation efficiency for PylRS WT and PylRS Y384F. They are both worked to incorporate MeaK with PylRS WT or PylRS Y384F. In our manuscript, we have applied intact protein mass spectrometry and tandem mass spectrometry to validate the incorporation fidelity of MeaK. Moreover, we have performed western blot analysis with pan-specific Kmea antibody (Figure R4).

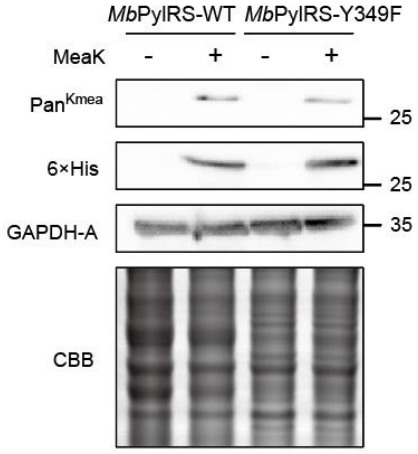

Figure R4: Comparison of MeaK incorporation efficiency between *Mb*PylRS Y384F and *Mb*PylRS WT. Western blot shows MeaK incorporated EGFP D190TAG by *Mb*PylRS Y384F or *Mb*PylRS WT, immunpblotting with anti-6×His antibody or anti-Kmea antibody. The loading control was E. coli whole cell lysates analyzed by Coomassie brilliant blue (CBB) or immunpblotting with anti-GAPDH-A antibody.

10. Microscopy images: almost all microscopy images are not visible and it is a really hard task to see what one should see. Please provide improved images.

Response: For the microscopy images, we are not aimed to show single cell. We want to show the fluorescence of cell populations.

Response:
I do not see the reason why not to show a magnification so that it is possible to see single cells.

Response: We thank the reviewer for his/her comments. The fluorescence intensity of cells is variable, a single cell can not precisely reflect the incorporation efficiency. It is important to show the cell populations here.

11. Can you provide a ESI-MS molecular weight determination of the whole MBP-Z protein? That will show if the protein is quantitatively Kmea-ylated. For EGFP-Y15Kmea (sometimes the authors write Kmea, sometimes MeaK, please be consistent) the authors also detect EGFP-Y15K protein (Fig. 1h). Can the authors explain how this was occurring? Does the improved PylRS-variant also charge PylT with K?
Response: The mass spectrum of integrated MBP-Z is shown in Figure R11. We apologize for the confusion. MeaK is the ncAA of ε-N-Methacryllysine, while Kmea is the type of protein modification. The EGFP Y151K may be due to demethacrylation of EGFP Y151MeaK by endogenous deacetylases.

Response:
Please provide the expected molecular weight and the obtained molecular weight. Are the data consistent? If not, explain (also in the revised manuscript).

Response: The theoretical molecular weight of MBP-Z E24MeaK is 50893.21 Da, the detected mass is 50893 Da, the data are consistent.

12. The authors consistently write “upregulated”/”downregulated” for the MS-IP data. This is wrong. The authors do not perform gene expression studies. It only means that they either identified proteins interaction stronger with one variant or better with another variant. In that context it does not become clear why the authors only focused on the improved interactions, while they did not say anything about proteins, which bind stronger to Cyp WT compares to the Kmea-ylated proteins.
Response: To avoid the confusion, we have changed ‘upregulated proteins’ and ‘downregulated proteins’ to ‘upregulated hits’ and ‘downregulated hits’, as shown in the manuscript. The expression of CypA K125MeaK is less than CypA WT and CypA K125A. Under this situation, the upregulated hits are really upregulated. For the downregulated hits, we can not differentiate that they are real downregulated hits or caused by low expression level of CypA K125MeaK. So, we focused on the upregulated hits.

Response:
I do not understand why using “upregulated hits/downregulated hits” is better compared to “upregulated/downregulated proteins”. My point was that this expression, i.e. upregulation

and downregulation, is wrong in this type of experiment. This is not an proteomics study looking at how gene expression affects protein level. Instead it is a pull down, where you see if proteins interact with your bait protein dependent on the presence of the modification. Please correct.

Response: We thank the reviewer for his/her comments. This is the general description in protein interaction study in proteomics field. If we used other description, this will cause readers confusion.

13. Fig. S12. The SIRT2 blot suggests unspecific binding.
Response: We agree with the reviewer that SIRT2 bind unspecifically with the beads. However, the binding of SIRT2 to CypA conjugated beads is stronger than the beads, suggesting that SIRT2 also bind with CypA.

Please confirm the direct interaction in a separate experiment (either in vitro or IP from cells using antibodies against SIRT2 or CypA (best would be at endogenous level and no overexpression!) instead of affinity enrichment with IMAC-bead or the beads where the authors observed unspecific binding of SIRT2.

Response: Due to the non-specific binding of SIRT2, we have removed the SIRT2 data from our manuscript.

14. It is not clear why the authors used the technique with the self-cleavable peptide. This needs a better description.
Response: We used mCherry proteins as an internal control. The self-cleavable peptide allows mCherry and EGFP to separate and fold independently after translation.

Response:
Please explain it in the manuscript in the materials and methods section.

Response: We have added this part to the methods section.

15. Fig. 4. The axes lack units.
Response: The y-axis counts the number of cells and the x-axis is the relative intensity of the fluorescent signal of FITC. The format of Fig.4 is same as the published figures in this paper (Huang L. et al. Immunity, 2021).

Response:
It is excellent that the axes is labelled as shown in the cited paper, please be sure that all figures throughout in the manuscript have axes labelled.

Response: We have carefully checked all of the figures.

16. Fig. S2: a loading control is missing. The legend is rudimental.

Response: We thank the reviewer for pointing this out, we have prepared this Figure again and rewritten the figure legend (Figure R13).

Response:
CBB staining is not a good loading control. Was that a CBB staining of the SDS-PAGE gel after blotting, i.e. showing reminiscent protein in the gel that was not transferred to the membrane? Or is the CBB stained gel a separate gel? Anyway, it is not a good loading control for an immunoblotting, so please state in the manuscript in the figure legends of all figures showing CBB staining as loading control what the loading control is and how that was done.

Response: CBB stained gel is a separate gel. We have explained the loading control in figure legend.

17. Line 156: “Stronger binding affinity was detected”. How was that determined?
Response: We have changed the description of ‘Stronger binding’ to ‘Stronger interaction’.

Response:I do not get the point why “stronger binding” is different and better compared to “stronger interaction”.

Response: Here, we used label-free quantitative mass spectrometry analysis to quantify the protein interactions. It is a routine strategy to quantify protein interactions.

18. The authors present a model according to which Kmea-ylatio of CypA affects ROS production by interfering with CsA binding. Did the authors experimentally measure if Kmea-ylation of CypA affects CsA binding?
Response: We thank the reviewer for his/her comments. We have already answered this question in an earlier comment. As demonstrated by MST, methacrylation of CypA affects CsA binding (Figure R8).

Response:
No answer was supplied concerning the impact of CypA on ROS production. Please comment and state in the manuscript how you measured the impact of CypA modification on ROS production and show the data.

Response: We thank the reviewer for his/her comments. The Figure 4 is how we measured the impact of CypA modification on ROS production.

19. What are “single-chain construct of CypA” (line 178), please explain.
Response: Single-chain means that we constructed the gene sequences of both CypA and mCherry proteins in the same plasmid and co-expressed them in one ORF.

Response:
The authors cannot express it this way. “Single-chain” is used for antibodies (as an

example). The term “chain” is used for proteins and not for DNA constructs. Please be sure that you use correct scientific language.

Response: We thank the reviewer for his/her comments. We have deleted all the “single-chain” in the manuscript.

Reviewer #3 (Remarks to the Author):

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

Reviewer #4 (Remarks to the Author):

The authors have addressed most points and made the manuscript easier to understand. Therefore, I consider the paper acceptable for publication.

Reviewer #2 (Black: reviewer comments, Blue: our response)

The revised version of the manuscript improved. Here are my concerns which were not sufficiently answered.

1. In the discussion is written:

"Methacrylation is an enzyme catalyzed biological process, and HAT1 and SIRT2 have been identified as methacryltransferase and de-methacrylase, respectively. Methacrylyl-CoA is the metharyl-group donor used by methacryltransferase to form Kmea.16 As a metabolite in the mitochondrial catabolism of valine, Methacrylyl-CoA is converted by ECHS1 to 3-hydroxyisobutyryl-CoA, which is further converted to 3- hydroxyisobutyrate by HIBCH. Deficiencies of ECHS1 and HIBCH will cause accumulation of Methacrylyl- CoA and lead to Leigh or Leigh-like syndrome"

Along to the point addressed earlier, from my point of view this suggests that HAT1 is the acyltransferase and SIRT2 the deacetylase. The authors do not state at this point that HDAC1 is also capable to act as de-methacrylase as they found here. However, these enzymes are not present in the mitochondria. So, it is totally unclear how methacrylation occurs in the mitochondrial matrix, either enzymatically or non- enzymatically. The authors should clearly state that it is unclear how methacrylation and de- methacrylation occurs in the mitochondria, i.e. if any mitochondrial acyltransferase (there is just one described in the literature) or deacetylase, i.e. SIRT3, SIRT4, SIRT5 can de-methacrylate proteins. The fact that the method is not good for mapping acyltransferases does mean that it is not suitable to experimentally identify these enzymes. However, this does not mean the authors cannot discuss the point on the regulation of this PTM in cells and to clearly say what is known and what not, i.e. it could be enzymatically or not-enzymatically catalyzed, but this is an unresolved question how it is regulated in mitochondria.

Response: We thank the reviewer for pointing these out. We agree with the reviewer. The processes of methacrylation and de-methacylation in mitochondria is important. We clarified the unresolved questions you pointed out and highlight them in the discussion section. In the future, we will systematically study the methacrylation and de-methacylation in mitochondria.

2. Statement:

"The MbPylRS (Y349F)/MbtRNApylCUA pair showed the highest incorporation efficiency of MeaK and, thus, was selected to incorporate MeaK into a model protein MBP-Z. An MBP-Z construct containing a TAG codon at site 24 and a C-terminal His-tag was coexpressed with the MbtRNApylCUA/MbPylRS (Y349F) pair in E. coli DH10B cells to successfully produce the full-length MBP-Z protein in the presence of MeaK (Fig.2c)."

Along the line of my earlier comments, I do not really see that *Mb*PylRS Y349F shows a higher incorporation efficiency compared to PylRS WT. If. I see correctly they only did it once for the EGFP-construct shown in the supplementary data, which did not have a proper loading control. Can the authors quantify this, please. I think both PylRS WT and PylRS Y349F have a similar efficiency of

incorporation. Do the authors see a difference in specificity of incorporation of Kmea into proteins using PylRS WT compared to PylRS Y349F? For PylRW WT it is known that it is rather promiscuous accepting many different amino acids.

Response: We thank the reviewer for his/her comments. We have carefully quantified the incorporation efficiency of *Mb*PylRS Y349F and *Mb*PylRS WT in three biological replicates (Figure R1). *Mb*PylRS Y349F and *Mb*PylRS WT showed similar incorporation efficiency. However, as the reviewer mentioned, *Mb*PylRS WT is known to accept a broad range of substrates. Based on intact mass and tandem mass spectrometry analysis, *Mb*PylRS Y349F showed high incorporation fidelity (Figure 2d). Therefore, we choose *Mb*PylRS Y349F to incorporate MeaK. We have revised the main text to clarify this point.

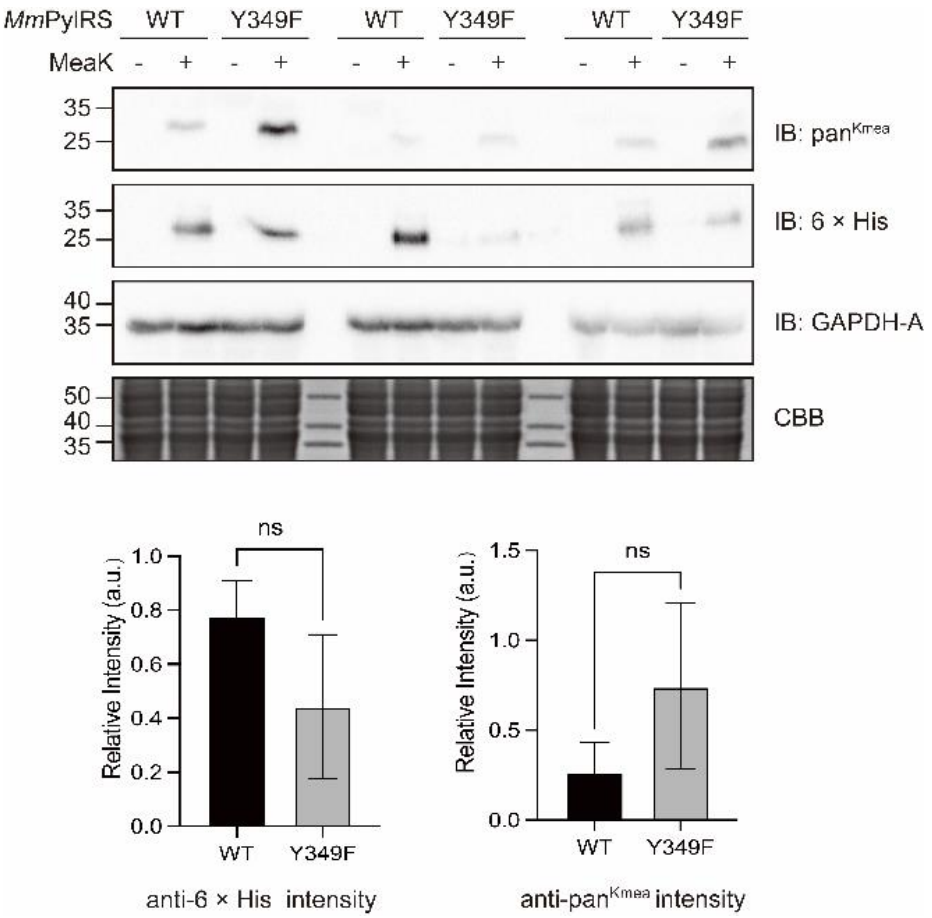

Figure R1: Comparison of MeaK incorporation efficiency between MbPylRS Y349F and MbPylRS WT. Western blot shows MeaK incorporated EGFP D190TAG by MbPylRS Y349F or MbPylRS WT, immunpblotting with anti-6×His antibody or anti-Kmea antibody. The loading control was shown by Coomassie brilliant blue (CBB) staining. The intensity of anti-6×His and anti-Pan^{Kmea} were separately normalized by intensity of GAPDH-A. Three biological replicates were performed.

3. References:

CypA was identified to be lysine acetylated at K125 in a paper back in 2006 by Kim et. al. This should be cited as I think it is important to highlight this lysine is reactive and accessible for different acylations. Paper: Kim, Sung Chan et al. Molecular Cell, Volume 23, Issue 4, 607 – 618

Response: We thank the reviewer for his/her comment. We have cited this paper.

4. Point or cirticism: Experiment in shown in Fig. R1, R4, and the identification of endogenously methacrylated CypA and DHRS2 protein.

The authors do not understand what I wanted to say with the point. I wanted to state that it might not be sufficient to state results in this manuscript, which are not part of this manuscript as there is not a real possibility to judge the experiments shown in the [redacted] paper if it is not published yet. If sho wing a systemic proteomics SILAC-based study in the [redacted] paper, why not just showing the dat a for thetwo proteins CypA and DHRS2 here in this manuscript? It is important to judge the physiolog ical significance that CypA and DHRS2 are Kmea-modifed at the endogenous level, i.e. without over expression.

Response: We thank the reviewer for his/her comments. The current manuscript focuses on the technology and application of incorporating methacryllysine into proteins. The study of endogenous lysine methacrylation is the focus of another paper currently under review in Cell Report. To answer the review's question about endogenous lysine methacrylation and its physiological significance, we have included the Cell Report manuscript in which we show endogenous Kmea modifications of CypA and DHRS2 in SILAC-based proteomic studies. We also show that absence of HIBCH or ECHS1 in cultured cells led to abnormal mitochondrial morphology and defects in respiration.

5. Avoid abbreviations such as "haven't".

Response: We apologize for this mistake his/her comment. We have corrected it.

6. Proint: Question regarding conversion of Kmea to Kmemea. The authors state now that p300 is a methyltransferase. To my knowledge, p300 is a lysine acetyltransferase only and is not capable to act as methyltransferase.

Response: We apologize for this mistake. We have corrected it.

7. Point MS data: I do not really see that there are more proteins present in the pull downs of modified CypA compared to WT or K125A mutant. Instead, it seems that the number is almost equal or even less for CypA Kmea compared to WT. the authors state that if they see more protein by MS there is higher binding affinity (or stronger binding). By the way, I still did not get the point what the difference is. Anyway, this is only true if for both samples the amount of protein, i.e. expression level, was the same. If you see an interaction in one sample and do not see it in the other it could also be due to differences in the expression level. Therefore, the authors should confirm that differences in the expression level was considered and the data were normalized to the expression level.

Response: We thank the reviewer for his/her comments. The purpose of this experiment is to detect changes in protein-protein interactions after Kmea modification. As suggested by the reviewer, we have changed "stronger binding" to "were enriched" in the maintext. Our data were normalized to the expression level as described in Bioinformatics Analysis in the methods section. We are aware of the difference in CypA protein expression level after Kmea incorporation. To avoid false positive results, we focus only on enriched proteins identified in the IP experiment with Kmea modified CypA, which had a lower expression level. We believe those enriched protein interactors for less expressed CypA Kmea are high-fidelity candidates as enhanced protein-protein interaction partners after Kmea modification.

8. As stated earlier, I recommend writing “Western blot” with capital “W”. At least make it consistent.

Response: We thank the reviewer for pointing this out. We have corrected it.

9. Why not stating the expected and calculated MW for the modified proteins as determined by ESI-MS. Here in the response letter the authors state the MWs but not in the manuscript. Please also provide these data for all recombinantly expressed and purified proteins, in addition to the Western blot using anti-Kmea antibody and the LC-MS/MS data to confirm the correct site for incorporation.

Response: We thank the reviewer for his/her comments. In this manuscript, we expressed and purified methacrylated MBP-Z (from E.coli) and EGFP (from HEK293T cells). We have stated the calculated MW of proteins in maintext. For MBP-Z, we provided the intact mass analysis (Fig. 2d), Western blot (Supplementary Fig. 3a), and tandem MS (Supplementary Fig. 3b). For EGFP were confirmed by intact mass data (Fig. 2i), Western blot (Supplementary Fig. 3a) and tandem MS (Fig. 2j).

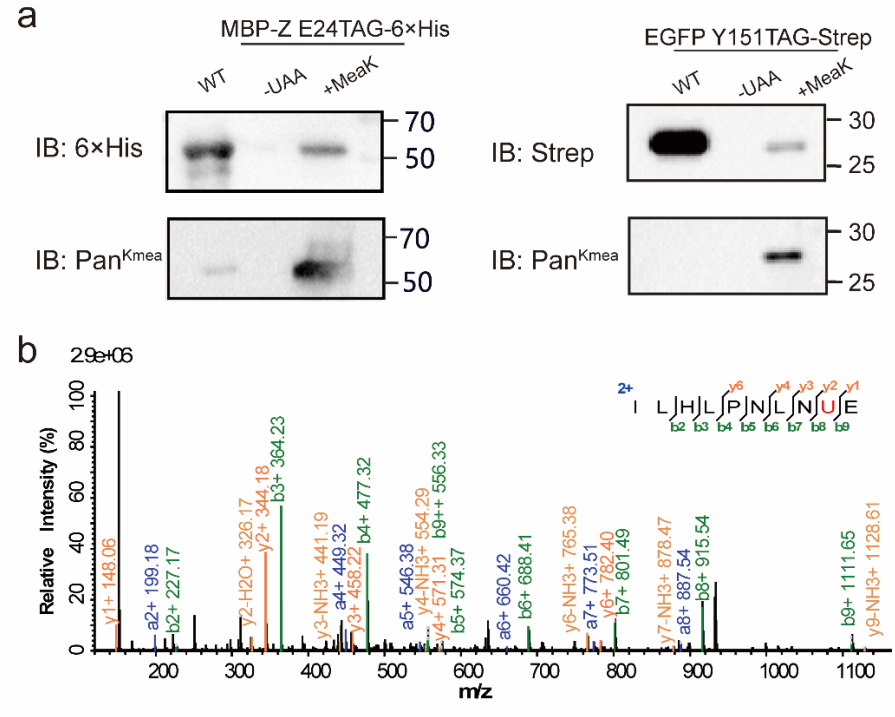

Figure R2: (a). Western blot of purified wild type MBP-Z and EGFP or mutant proteins with or without MeaK incorporated. MBP-Z was purified from E. coli though 6 × His tag and EGFP was purified in HEK293T cells though Strep tag. (b). Tandem mass spectrum of MeaK incorporated peptide of MBP-Z E24MeaK. The red U represents MeaK incorporation site.

10. Upregulated/downregulated in proteomics: I think it is not a general description for protein-protein interactions in proteomics. It is used for expression-proteomics, i.e. analyzing differences in expression levels as gene expression is up- or downregulated. In a protein-protein interaction nothing is regulated in the interaction in terms of a gene expression. Instead, the authors should ensure to normalize for the expression level in case the protein of interest affects gene expression. Even if you find papers saying “up- or downregulation” for analyzing protein-protein interactions, this does not make it more

correct. For a protein-protein interaction you should use the expressions “enriched” or “depleted” or similar, assuming expression levels are similar and the formation or abolishment of the respective interaction only depends on the presence of the modification of the bait protein.

Response: We thank the reviewer for his/her comments. We have changed the “upregulated” or “downregulated” to “enriched” or “depleted”.

11. Labelling of axes: still not all axes are labelled. Please check Fig. 4a,b,c,d. What is on the x-axis? FTIC is the abbreviation for fluoresceine isothiocyanate but what are the units shown here? What unit does the y-axis have (count)? What unit is shown on the y-axis in Fig. 4d?

Response: We thank the reviewer for his/her comments. We have corrected labels in Fig. 4. The Y-axis shows the number of cells examined and the unit is n. X-axis shows the DCFH-DA fluorescence intensity detected using the FITC channel (488 nm laser for excitation and 525/40 bandpass (BP) for fluorescence detection). The values were from the original data output by the flow cytometer with arbitrary units (a.u.). These values are relative values that depend on the setting of the instrument and are not standardized (https://pubs.acs.org/doi/10.1021/acssynbio.5b00284 Castillo-Hair SM. et.al., ACS Synth Biol. 2016). The Y-axis of Fig. 4d shows the mean fluorescence intensity in the same unit as Fig. 4a, b, c.

12. Interactions: it would be very good if the authors could validate a few interactions, such as the interaction with thioredoxin, of modified CypA and the identified interaction partners by other methods, i.e analytical SEC, IP, MST, ITC or SPR, etc.

Response: We thank the reviewer for his/her comments. As suggested, we validated the interaction between thioredoxin and CypA-K125MeaK using co-IP. The recombinant TXN1 plasmid (TXN1-6xHis) was co-transfected into HEK293T cells with the CypA plasmid (CypA-K125Meak-Strep). After cell lysis, we performed immunoprecipitation using the anti-His antibody. Western blot analysis showed the pulldown of CypA-K125MeaK (Figure R3). The co-IP experiment verified the interaction between thioredoxin and CypA-K125MeaK.

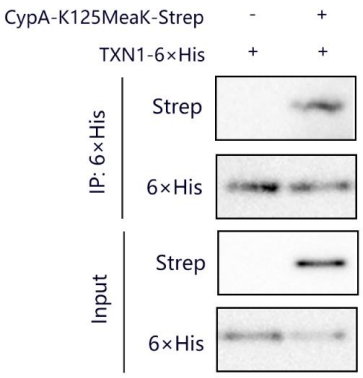

Figure R3: Co-IP experiment validated the protein interaction between thioredoxin 1 (TXN1) and Kmea modified CypA (CypA-K125MeaK).

The revised version of the manuscript improved. Here are my concerns which were not sufficiently answered.

1. In the discussion is written:

"Methacrylation is an enzyme catalyzed biological process, and HAT1 and SIRT2 have been identified as methacryltransferase and de-methacrylase, respectively. Methacrylyl-CoA is the metharyl-group donor used by methacryltransferase to form Kmea.16 As a metabolite in the mitochondrial catabolism of valine, Methacrylyl-CoA is converted by ECHS1 to 3-hydroxyisobutyryl-CoA, which is further converted to 3-hydroxyisobutyrate by HIBCH. Deficiencies of ECHS1 and HIBCH will cause accumulation of Methacrylyl-CoA and lead to Leigh or Leigh-like syndrome"

Along to the point addressed earlier, from my point of view this suggests that HAT1 is the acyltransferase and SIRT2 the deacetylase. The authors do not state at this point that HDAC1 is also capable to act as de-methacrylase as they found here. However, these enzymes are not present in the mitochondria. So, it is totally unclear how methacrylation occurs in the mitochondrial matrix, either enzymatically or non-enzymatically. The authors should clearly state that it is unclear how methacrylation and de-methacrylation occurs in the mitochondria, i.e. if any mitochondrial acyltransferase (there is just one described in the literature) or deacetylase, i.e. SIRT3, SIRT4, SIRT5 can de-methacrylate proteins. The fact that the method is not good for mapping acyltransferases does mean that it is not suitable to experimentally identify these enzymes. However, this does not mean the authors cannot discuss the point on the regulation of this PTM in cells and to clearly say what is known and what not, i.e. it could be enzymatically or not-enzymatically catalyzed, but this is an unresolved question how it is regulated in mitochondria.

2. Statement:
"The MbPylRS (Y349F)/MbtRNA$_{pylCUA}$ pair showed the highest incorporation efficiency of MeaK and, thus, was selected to incorporate MeaK into a model protein MBP-Z. An MBP-Z construct containing a TAG codon at site 24 and a C-terminal His-tag was coexpressed with the MbtRNA$_{pylCUA}$/MbPylRS (Y349F) pair in E. coli DH10B cells to successfully produce the full-length MBP-Z protein in the presence of MeaK (Fig. 2c)."

Along the line of my earlier comments, I do not really see that *Mb*PylRS Y349F shows a higher incorporation efficiency compared to PylRS WT. If. I see correctly they only did it once for the EGFP-construct shown in the supplementary data, which did not have a proper loading control. Can the authors quantify this, please. I think both PylRS WT and PylRS Y349F have a similar efficiency of incorporation. Do the authors see a difference in specificity of incorporation of Kmea into proteins using PylRS WT compared to PylRS Y349F? For PylRW WT it is known that it is rather promiscuous accepting many different amino acids.

3. References:
CypA was identified to be lysine acetylated at K125 in a paper back in 2006 by Kim et. al. This should be cited as I think it is important to highlight this lysine is reactive and accessible for different acylations.
Paper: Kim, Sung Chan et al. Molecular Cell, Volume 23, Issue 4, 607 - 618

4. Point or cirticism: Experiment in shown in Fig. R1, R4, and the identification of endogenously methacrylated CypA and DHRS2 protein.

The authors do not understand what I wanted to say with the point. I wanted to state that it might not be sufficient to state results in this manuscript, which are not part of this manuscript as there is not a real po ssibility to judge the experiments shown in the [redacted] paper if it is not published yet. If showing a syst emic proteomics SILAC-based study in the [redacted] paper, why not just showing the data for the

two proteins CypA and DHRS2 here in this manuscript? It is important to judge the physiological significance that CypA and DHRS2 are Kmea-modifed at the endogenous level, i.e. without overexpression.

5. Avoid abbreviations such as “haven’t”.

6. Proint: Question regarding conversion of Kmea to Kmemea. The authors state now that p300 is a methyltransferase. To my knowledge, p300 is a lysine acetyltransferase only and is not capable to act as methyltransferase.

7. Point MS data: I do not really see that there are more proteins present in the pull downs of modified CypA compared to WT or K125A mutant. Instead, it seems that the number is almost equal or even less for CypA Kmea compared to WT. the authors state that if they see more protein by MS there is higher binding affinity (or stronger binding). By the way, I still did not get the point what the difference is. Anyway, this is only true if for both samples the amount of protein, i.e. expression level, was the same. If you see an interaction in one sample and do not see it in the other it could also be due to differences in the expression level. Therefore, the authors should confirm that differences in the expression level was considered and the data were normalized to the expression level.

8. As stated earlier, I recommend writing “Western blot” with capital “W”. At least make it consistent.

9. Why not stating the expected and calculated MW for the modified proteins as determined by ESI-MS. Here in the response letter the authors state the MWs but not in the manuscript. Please also provide these data for all recombinantly expressed and purified proteins, in addition to the Western blot using anti-Kmea antibody and the LC-MS/MS data to confirm the correct site for incorporation.

10. Upregulated/downregulated in proteomics: I think it is not a general description for protein-protein interactions in proteomics. It is used for expression-proteomics, i.e. analyzing differences in expression levels as gene expression is up- or downregulated. In a protein-protein interaction nothing is regulated in the interaction in terms of a gene expression. Instead, the authors should ensure to normalize for the expression level in case the protein of interest affects gene expression. Even if you find papers saying “up- or downregulation” for analyzing protein-protein interactions, this does not make it more correct. For a protein-protein interaction you should use the expressions “enriched” or “depleted” or similar, assuming expression levels are similar and the formation or abolishment of the respective interaction only depends on the presence of the modification of the bait protein.

11. Labelling of axes: still not all axes are labelled. Please check Fig. 4a,b,c,d. What is on the x-axis? FTIC is the abbreviation for fluoresceine isothiocyanate but what are the units shown here? What unit does the y-axis have (count)? What unit is shown on the y-axis in Fig. 4d?

12. Interactions: it would be very good if the authors could validate a few interactions, such as the interaction with thioredoxin, of modified CypA and the identified interaction partners by other methods, i.e analytical SEC, IP, MST, ITC or SPR, etc.