nature portfolio

Peer Review File

Development of nucleus-targeted histone-tail-based photoaffinity probes to profile the epigenetic interactome in native cells

Corresponding Author: Professor Yi-Ming Li

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

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Identification of the protein-protein interactions mediated by histone PTMs is a long-standing research interest in the field of epigenetics and is crucial for investigating epigenetic events. Approaches have been developed to tackle various challenges in profiling the interactomes of histone PTMs, and they indeed largely facilitate the understanding of epigenetic regulation. In this manuscript, Jian Fan et al developed a method that can deliver photoaffinity peptide probe into nucleus of live cells, thus enabling the identification of histone PTM-mediated protein interactions in a native context. This approach straightforwardly integrated photo-reactive group, nuclear localization sequence, cell-penetrating peptide, and biotin into histone PTM-based peptide probe, with careful optimization in terms of cell-permeability and nuclear localization. Then the authors profiled the interactomes of H3K4me3 in live HeLa and RAW 264.7 cells for proof-of-concept, and investigate the interactions mediated by H3K9la, a newly identified histone PTMs which is implicated to regulate epigenetic event.

Overall, the idea of this manuscript is attractive. The part to optimize the cell-permeability and nuclear localization is sound (Fig. 2 and related supp. Figures), and capability of the probes had been well demonstrated in vitro (Fig. 3 and related supp. Figures). However, the investigation of histone PTMs interactomes in live cells (Fig. 4-6), which is the key point of this manuscript, is problematic, thus preventing me from supporting its publication in Nature Communication.

1. The major concern is that the authors failed to demonstrate the capability of this method in efficiently and specifically profiling the interactomes of histone PTMs in live cells. Judging from the proteomic data obtained by the developed method (Fig. 4-Fig. 5), it could not distinguish the proteins recognizing H3K4me3 and H3K4me0 and reliably identify them from cellular context.
For example, if use a cutoff of FC > 1.5 as usually applied in quantitative proteomics (e.g., the Burton’s study cited as ref. 21 in this manuscript), only 5 known H3K4me3-interactors (TAF3, KDM2A, KDM5A, CXXC1, DIDO1) were specifically enriched by H3K4me3 probe in this study. It is quite strange that the authors used such a low cutoff (FC > 1) in the manuscript, and I presume that this is because that they wanted to include as much as possible known H3K4me3-interactors (20 if use FC > 1). However, such low cutoff is problematic and significantly reduces the reliability of the methods developed in this manuscript.

If look into the MS dataset, the FC values of many well-characterized H3K4me3 ‘readers’/‘eraser’ obtained in this study were very low (some of them even cannot be considered as “enriched”), compared to the two studies cited (ref. 15 and ref. 21; Figure 4d, Table S2). For example, a well-known and very tight binder of H3K4me3, SPIN1, only showed FC = 1.33 (HeLa) and 1.40 (RAW) in this study, while it has FC = 15.3 (Burton et al; ref 21) or FC = 4 (Lin et al; ref 15) in others. And PHF8 (a well-characterized H3K4me3 ‘eraser’) displayed FC value of only 1.23 in this study, while 4.14 and 4.92 in Burton’s and Lin’s studies, respectively. Importantly, 9 out of 11 known interactors that were only ‘identified’ in this study (Fig. 4d, Fig. S22), which may potentially demonstrate its capability over others, also showed very low FC value (supp. dataset 1), namely PHF6 (1.07), CHD9 (1.12), ORC3 (1.34), PHF5A (1.05), CHD8 (1.18), WDR81 (1.14), WDR3 (1.06), WDR11 (1.16), PHF20L1 (1.15).

Even if the author thought it is necessary to use such low cutoff because of some special experimental settings or conditions, then it raises another significant concern about the high false-positive rate. Using the current cutoff (FC > 1; P value < 0.05),

297 identified proteins were considered as H3K4me3-specific interactors, while only 20 of them are known binders (7%). Among the remaining 277, how many of them could be potential novel H3K4me3-interactors and how many of them are false positive? This will cause lots of problems when applying this approach to uncharacterized PTMs. As a comparison, in Burton's study (ref. 21; FC > 1.5; P value < 0.05), 19 out of 25 (76%) identified H3K4me3-specific binders were known interactors.

Moreover, present approach seemed not capable of identifying the proteins excluded by H3K4me3 marks. For example, some known H3K4me0-specific binders, RBBP4, MTA1/2, were not identified in this study, while identified in both ref.15 and ref. 21. Therefore, the proteomic data is not convincing to demonstrate the capability of the approach to profile the interactomes of histone PTMs.

2. Because of the abovementioned concern, the profiling of H3K9la interactome is also not reliable, especially considering that totally only ~130 proteins were identified from proteomic study, and that the FC value for AF9 is only 1.36, while several hits with higher FC value and lower P value were not verified.

3. Judging from the LC-MS data, the purity of some probes (including some probes used in proteomic experiments) is not good, which may seriously affect their behaviors in cells and maybe the reason of unsatisfactory proteomic results. To be more specific:
1) LC trace contains significant impurity: Fig. S2a, S3b, S4b, S5b, S7a, S9a, S11b, S12b, S28a.
2) ESI-MS shows more than one set of peaks: Fig. S5c, S12c, S15b, S16c, S26c, S28b.

Reviewer #2

(Remarks to the Author)
This work led by Dr. Li's and Dr. Liu's teams developed a set of cell-penetrating, nucleus-targeted histone photoaffinity probes to identify the interactomes of histone post-translational modifications (hPTMs). Cell-permeable and nuclear localization peptide modules were built into the modified peptide, which allows nuclear localization in native cells. Using this probe, the authors were able to identify putative binders of H3K4me3, and dozens of them were well-established H3K4me3 reader proteins, which is on par with previous studies. Next, they applied the same probe to hard-to-transfect macrophage RAW264.7 cells, which yielded similar results and highlighted the potential applicability of this tool in technically challenging cell types. Furthermore, the authors designed a new probe targeting a recently discovered hPTM, H3K9la. From the quantitative mass spectrometry results, they characterized AF9 as a H3K9la binder. This suggests the possibility of this approach in identifying binders of understudied types of hPTMs.
Overall, the present study has provided high-quality data to support their main conclusions. The new probes may be of interest to the research community for PTMs and has the potential to identify more binders of diverse PTMs. There are only a few concerns to be addressed as listed below.
1. Fig. 3d and 3e. It seems that probe 10 could bind SPIN1 and KDM4A to a lower degree than probe9. This raises the question of false positive and false negative identification. The authors are expected to comment on how non-specific interactions compromise the identification of hPTM binding proteins. For example, are there proteins in Fig. 4b that are well-established H3K4me3 binding proteins but not enriched in the quantitative proteomics result due to binding to probe 4 (false negative proteins)?
2. You mentioned in your paper that 20 previously reported H3K4me3 readers were identified in your assay. Are these direct binders or the total number of proteins involving both direct and indirect binding proteins. Please elaborate on what percentage of known H3K4m3-binders were recovered in your assay.
3. Please include the confocal microscopy images of RAW264.7 cells treated with probes.
4. Fig. 6. When comparing the results from Fig. 5 and Fig. 6, one would wonder why les proteins were identified using probes 12 and 13. Are there proteins overlapping between Fig. 5a and Fig. 6c?
5. Putative histone lactylatio binding proteins were identified in this datasets. It would be ideal if the authors could validate additional proteins.
6. Given the promising results, one would wonder if the presented approach could be used for quantitative studies, i.e. profiling differential hPTM binding proteins between distinct conditions.
7. There are a few typos and grammatical errors in the manuscript.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
In the revised manuscript, the authors have made commendable efforts to improve their proteomic data, including the use of LFQ to replace the previous TMT experiments and the addition of a proteomic study profiling H3K9cr binding proteins to showcase the generalizability of their strategy. Many of my previous concerns have been addressed. However, a few issues remain that I believe still need attention:
1. Compared to the previously developed protein trans-splicing strategy that incorporates both the photo-affinity group and

post-translational modifications (PTM) into native chromatin, the current probes are based on histone tail peptides. This approach cannot fully recapitulate the native chromatin environment, and this limitation should be discussed in the manuscript. Additionally, the term “histone photoaffinity probe” may be misleading, and I suggest using “histone-tail-based photoaffinity probe” to better reflect the nature of the probes.
2. LC-MS Data: I appreciate the authors' explanation for the additional peaks observed in their LC traces. However, some of the ESI-MS spectra still appear unclear. While I understand that certain peaks result from the loss of N2 and the TFA adducts, there are additional unassigned peaks (notably in Figures S5C, S16C, S26B, and S27C) that resemble impurities. It would be beneficial if the authors could repurify these probes and provide cleaner spectra to improve the overall quality of the data.
Minor Issues:
• Figures 4d, 4e, and 5f: I recommend the authors double-check the references in these figures, as some are not found in the reference section.

Reviewer #2

(Remarks to the Author)
The authors have addressed most of the comments raised by this reviewer. They have provided a decent amount of data that support their conclusions, and their arguments are well taken. Overall, the quality of the manuscript has been improved, so this reviewer would suggest publication if they can address the following comment.
Figure 6. Obviously AF9’s binding affinity to H3K9la is lower than the other types of acylations, such as acetylation. Even though ITC data in Figure 6c demonstrates this binding in vitro, more evidence is needed to consolidate it in cells. The authors are expected to provide more evidence to demonstrate that this finding is biologically relevant. For example, the authors could perform an IP experiment in Raw264.7 cells with or without sodium lactate treatment and compare the level of AF9 enrichment using confocal microscopy, WB, or mass spec.

Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have addressed all my concerns . I support the publication of this manuscript.

Reviewer #2

(Remarks to the Author)
The authors have addressed my concerns.

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Reviewer 1:

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Response: We appreciate your positive comments about our new concept probes.

1. The major concern is that the authors failed to demonstrate the capability of this method in efficiently and specifically profiling the interactomes of histone PTMs in live cells. Judging from the proteomic data obtained by the developed method (Fig. 4-Fig. 5), it could not distinguish the proteins recognizing H3K4me3 and H3K4me0 and reliably identify them from cellular context.

For example, if use a cutoff of FC > 1.5 as usually applied in quantitative proteomics (e.g., the Burton's study cited as ref. 21 in this manuscript), only 5 known H3K4me3-interactors (TAF3, KDM2A, KDM5A, CXXC1, DIDO1) were specifically enriched by H3K4me3 probe in this study. It is quite strange that the authors used such a low cutoff (FC > 1) in the manuscript, and I presume that this is because that they wanted to include as much as possible known H3K4me3-interactors (20 if use FC > 1). However, such low cutoff is problematic and significantly reduces the reliability of the methods developed in this manuscript.

Response: In our previous work, we used TMT-based quantitative chemoproteomics to identify interactors. However, according to previous reports, the quantitation accuracy of TMT is disturbed by a phenomenon referred to as ratio compression, which causes the FC value being skewed towards a 1:1 ratio (*Nat Methods* **2011**, *8*, 93; *Clin. Proteomics* **2022**, *19*, 13). Therefore, low cutoff can be used for TMT-based chemoproteomics in some special experimental settings, for example, Alice Y. Ting et al. used cutoff of FC > 1 when using TurboID and APEX in combination with TMT-based quantitative chemoproteomics to identify interactors (*Nat. Commun.* **2021**, *12*, 4980; *Cell* **2023**, *186*, 3307-3324).

In revised manuscript, to further demonstrate the reliability of our methods, we used label-free quantitative (LFQ) proteomics to analyze the H3K4me3 interactomes with a cutoff of FC > 1.5, which also revealed a significant enrichment of 18 and 11 established H3K4me3 interactors in HeLa and RAW264.7 cells, respectively (Fig. 1a and 1b). These results are also similar to the number of interactors obtained by previous works using the SILAC-based quantitative proteomics labeling approach to probing H3K4me3 interactomes.

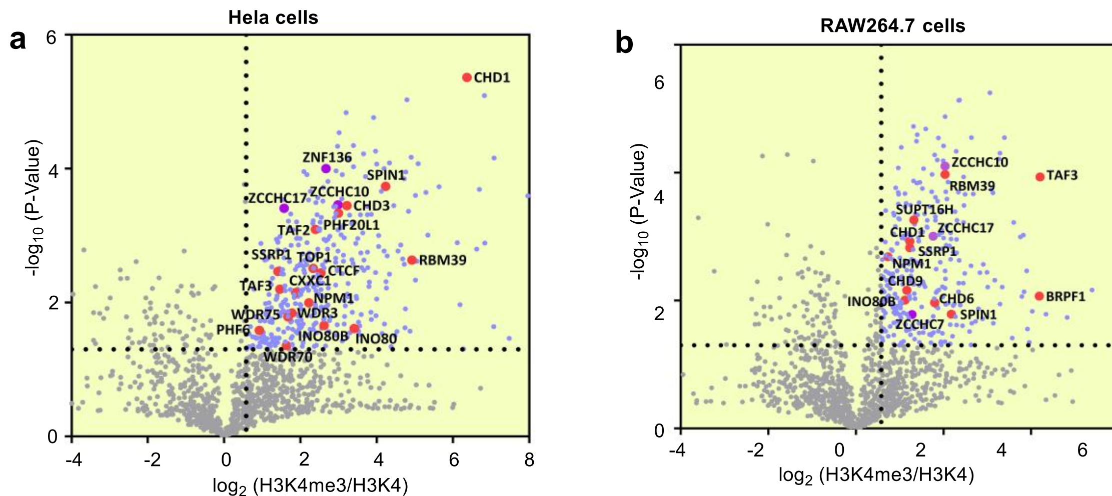

Fig. 1 a) Volcano plots of the quantitative mass spectrometry results in Hela cells. Significantly enriched hits ($p < 0.05$, >1.5-fold change) are colored blue. Some established H3K4me3 reader proteins are highlighted and labeled in red. b) Volcano plots of the quantitative mass spectrometry results in RAW264.7 cells. Significantly enriched hits ($p < 0.05$, >1.5-fold change) are colored blue. Some established H3K4me3 reader proteins are highlighted and labeled in red.

If look into the MS dataset, the FC values of many well-characterized H3K4me3 'readers'/'eraser' obtained in this study were very low (some of them even cannot be considered as "enriched"), compared to the two studies cited (ref. 15 and ref. 21; Figure 4d, Table S2). For example, a well-known and very tight binder of H3K4me3, SPIN1, only showed FC = 1.33 (HeLa) and 1.40 (RAW) in this study, while it has FC = 15.3 (Burton et al; ref 21) or FC = 4 (Lin et al; ref 15) in others. And PHF8 (a well-characterized H3K4me3 'eraser') displayed FC value of only 1.23 in this study, while 4.14 and 4.92 in Burton's and Lin's studies, respectively. Importantly, 9 out of 11 known interactors that were only 'identified' in this study (Fig. 4d, Fig. S22), which may potentially demonstrate its capability over others, also showed very low FC value (supp. dataset 1), namely PHF6 (1.07), CHD9 (1.12), ORC3 (1.34), PHF5A (1.05), CHD8 (1.18), WDR81 (1.14), WDR3 (1.06), WDR11 (1.16), PHF20L1 (1.15).

Response: Thanks for your suggestions. In the results of the profiling of H3K4me3-interactors using LFQ-based proteomics, the FC value of most known H3K4me3-interactors mentioned by reviewers was significant increased, for instance, the FC = 18.90 (HeLa cells) and 4.53 (RAW264.7 cells) for SPIN1 (Tudor) are comparable to previous results of FC = 15.3 (Burton et al; ref 23) or FC = 4 (Lin et al; ref 17); CXXC1 (PHD) showed FC = 3.67 (HeLa cells) in this study, also similar to previous results of FC = 3.35 (Burton et al) or FC = 3.5 (Lin et al). CHD1 (Chromeo), which showed FC =82.74 (HeLa cells) and 2.34 (RAW264.7 cells) in this study, which is significantly higher than the previous results FC = 1.80 (Burton et al) (Table 1).

Known H3K4me3-interactors	This study		Burton, A. J et al.	Li, j. W. et al
	HeLa	RAW		
SPIN1 (Tudor)	18.90	4.53	15.3	4
CXXC1 (PHD)	3.67	/	3.35	3.5
CHD1 (Chromeo)	82.74	2.34	1.80	/

Table 1 Comparison of FC values of our probe and the H3K4me3-interactors enriched by two recent studies.

Furthermore, other H3K4me3-interactors captured in present study also showed FC values higher than 1.5. In Hela cells: CHD3 (9.29), TAF2 (5.27), TAF3 (2.76), PHF6 (1.90), PHF20L1 (7.97), WDR3 (3.43), WDR70 (3.10), WDR75 (3.19), RBM39 (30.48), INO80 (10.67), INO80B(6.15), TOP1 (5.06), NPM1 (4.66), CTCF (5.82), SSRP1 (2.66); in RAW264.7 cells: TAF3 (18.36), CHD9 (2.23), CHD6 (3.47), BRPF1 (18.21), SSRP1 (2.35), SUPT16H (2.51), RBM39 (4.09), NPM1 (1.66), INO80B (2.17). In the revised manuscript, we have added the description of this section on Page 10, line 14-Page 10, line 28.

Moreover, to demonstrate the generalizability of our new strategy, we synthesized the histone H3 lysine 9 crotonylation (H3K9cr) probe in revised work, and performed interactome profiling for histone crotonylation in macrophage. By employing a cutoff of FC > 1.5 and p value < 0.05, we identified eight proteins that interact with H3K9cr, either directly or indirectly. Among these, DPF2 (DPF family) showed FC = 2.81, and Sirt1 ("eraser" enzymes) showed FC = 3.28 in the present study, similar to previously reported Fc values (DPF2 in 293T cells, *Anal. Chem.* **2022**, *94*, 10705-10714; Sirt1 in HeLa cells, *Elife* **2014**, *3,* e02999). Notably, other interactors including AF9 (YEATS family, FC = 3.24), Rps19bp1 (Active regulator of SIRT1, FC = 3.22), TAF2 (the subunits of the TFIID complex, FC = 1.82), TAF3 (the subunits of the TFIID complex, F C= 1.55), PHF11 (by similar to PHF10, FC = 3.75), PHF20L1 (by similar to PHF10, FC = 2.95), were only enriched by our probe. In the revised manuscript, we have added the description of this section on Page 14, line 3-Page 14, line 20.

Taken together, all the results confirmed the reliability of our methods to profile the epigenetic interactome in live cells.

Even if the author thought it is necessary to use such low cutoff because of some special experimental settings or conditions, then it raises another significant concern about the high false-positive rate. Using the current cutoff (FC > 1; P value < 0.05), 297 identified proteins were considered as H3K4me3-specific interactors, while only 20 of them are known binders (7%). Among the remaining 277, how many of them could be potential novel H3K4me3-interactors and how many of them are false positive? This will cause lots of problems when applying this approach to uncharacterized PTMs. As a comparison, in Burton's study (ref. 21; FC > 1.5; P value < 0.05), 19 out of 25 (76%) identified H3K4me3-specific binders were known interactors.

Response: Thanks for your suggestions. In addition to the utility the SILAC quantitative proteomics to analyze the histone interactome, TMT-based quantitative proteomics was also in previous work to profile H3.1 versus interactors (*Nature* **2023**, *616*, 574-580, Fig.2h and Supplementary Table 3). Using (FC > 1.4; P value < 0.05) as a cut-off, 243 proteins met the standard, but only 9 of those were known binding proteins (3.7%) (Fig. 2a). Moreover, the previous work also used proteomics of LFQ with a cutoff of (FC > 1.5; P value < 0.05) to identify H3K9cr-interactors (*Anal. Chem.* **2022**, *94*, 1070; Fig. 4C and Fig. S4B). This approach yielded 775 proteins that met the specified criteria, but only 21 of these proteins maybe binding proteins (2.7%) (Fig. 2b).

Taking together with these data and our results, we believe that the use of SILAC quantification proteomics may be the main reason for the lower false positives when probing for H3K4me3-interactors. The relative higher false positive rate of our results and other works (*Nature* **2023**, *616*, 574-580; *Anal. Chem.* **2022**, 94, 10705-10714) may be caused by the different quantitative methods of mass spectrometry. In the future study, we will utilize SILAC quantitative proteomics combined with our new probe to further explore the interactomes of histone PTMs *in situ*, and look forward to more in-depth results. In the revised manuscript, we have added the description of this section on Page 19, line 17-Page 19, line 22.

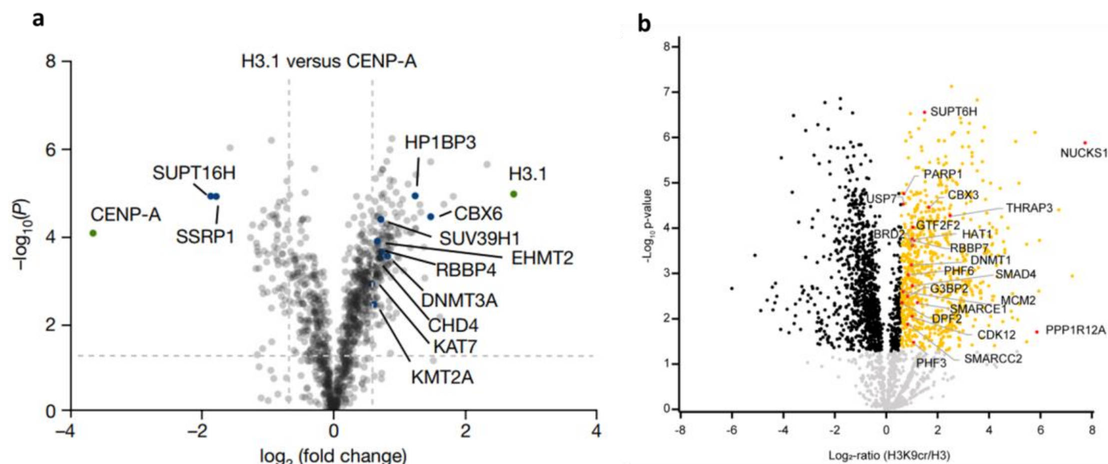

Fig. 2 a) Volcano plots of H3.1 versus interactors (*Nature* **2023**, *616*, 574). b) Volcano plots of H3K9cr-interactors (*Anal. Chem.* **2022**, 94, 10705).

Moreover, present approach seemed not capable of identifying the proteins excluded by H3K4me3 marks. For example, some known H3K4me0-specific binders, RBBP4, MTA1/2, were not identified in this study, while identified in both ref.15 and ref. 21. Therefore, the proteomic data is not convincing to demonstrate the capability of the approach to profile the interactomes of histone PTMs.

Response: In revised manuscript, we have identified some H3K4me0-specific binders, such as HAT1 and UHRF1 in HeLa cells; CAMTA1, MTA2, DPF2, and RBBP7 in RAW cells (Table 2)

Known H3K4me0 -interactors	FC(H3K4me3/H3K4me0)	
	HeLa	RAW
HAT1	0.56	/
UHRF1	0.55	/
CAMTA1	/	0.46
MTA2	/	0.53
DPF2	/	0.31
RBBP7	/	0.68

Table 2 H3K4me0-specific binders identified in HeLa cells and RAW cells.

2. Because of the abovementioned concern, the profiling of H3K9la interactome is also not reliable, especially considering that totally only ~130 proteins were identified from proteomic study, and that the FC value for AF9 is only 1.36, while several hits with higher FC value and lower P value were not verified.

Response: Combined with ITC results of AF9 (KD=191 μM), we consider that the weak interaction between AF9 and H3K9la may be the main reason for the low FC value. Notably, our result further suggests that, unlike the strong interaction of crotonylation with AF9, the epigenetic factor AF9 may be a "blunted recognition" reader of histone lactonylatio. Our preliminary study also indicates that this reader may play a key function in suppressing the formation of the tumor microenvironment (unpublished results).

We also analyzed other captured proteins and found that only four proteins had a higher FC value and a lower p value than AF9. Two of them are histones (H1-3, H1-5), one is a subunit of the large ribosome (Rpl36a), and another one is a thiol-specific peroxidase. The function of these proteins was not closely correlate with histone lactylation, so we did not perform the binding affinity tests.

3. Judging from the LC-MS data, the purity of some probes (including some probes used in proteomic experiments) is not good, which may seriously affect their behaviors in cells and maybe the reason of unsatisfactory proteomic results. To be more specific:
1) LC trace contains significant impurity: Fig. S2a, S3b, S4b, S5b, S7a, S9a, S11b, S12b, S28a.
Response: Thanks for your suggestions. These compounds all carry the fluorescent moiety rhodamine B (RhoB). Because RhoB has a reversible equilibrium between non-fluorescent spironolactone and fluorescent zwitterions (*Angew. Chem. Int. Ed.* **2023**, e202307641), thus producing two peaks in the chromatogram. This reason is explained in revised manuscript. In comparison, this does not occur with probes that do not carry RhoB, as shown in Fig. S15a (probe **9**), S16a (probe **10**).

2) ESI-MS shows more than one set of peaks: Fig. S5c, S12c, S15b, S16c, S26c, S28b.
Response: Thanks for your suggestions. Fig. S12c, Fig. S15b, Fig. S16c will lose N_2 when carrying the photocrosslinked group (diazirine) (*Chem. Sci.* **2015**, *6*, 1011-1017). We give an explanation in revised manuscript.

Reviewer #2
This work led by Dr. Li's and Dr. Liu's teams developed a set of cell-penetrating, nucleus-targeted histone photoaffinity probes to identify the interactomes of histone post-translational modifications (hPTMs). Cell-permeable and nuclear localization peptide modules were built into the modified peptide, which allows nuclear localization in native cells. Using this probe, the authors were able to identify putative binders of H3K4me3, and dozens of them were well-established H3K4me3 reader proteins, which is on par with previous studies. Next, they applied the same probe to hard-to-transfect macrophage RAW264.7 cells, which yielded similar results and highlighted the potential applicability of this tool in technically challenging cell types. Furthermore, the authors designed a new probe targeting a recently discovered hPTM, H3K9la. From the quantitative mass spectrometry results, they characterized AF9 as a H3K9la binder. This suggests the possibility of this approach in identifying binders of understudied types of hPTMs.
Overall, the present study has provided high-quality data to support their main conclusions. The new probes may be of interest to the research community for PTMs and has the potential to identify more binders of diverse PTMs. There are only a few concerns to be addressed as listed below.
Response: Thanks for your positive comments.

1. Fig. 3d and 3e. It seems that probe 10 could bind SPIN1 and KDM4A to a lower degree than probe9. This raises the question of false positive and false negative identification. The authors are expected to comment on how non-specific interactions compromise the identification of hPTM binding proteins. For example, are there proteins in Fig. 4b that are well-established H3K4me3 binding proteins but not enriched in the quantitative proteomics result due to binding to probe 4 (false negative proteins)?
Responses: Since probe **9** carries trimethylation modification and probe **10** does not, SPIN1 and KDM4A, which are known to bind H3K4me3, should preferably bind to probe **9**. In Fig. 3d and 3e (in the revised manuscript), we also demonstrated that probe **10** hardly forms cross-linking bands with SPIN1 and KDM4A, while probe **9** forms cross-linking bands with SPIN1 and KDM4A. To compare the conjugation efficiency of probe **9** and probe **10**, we calculated the (band intensity of probe **10**-SPIN1 conjugate)/ (band intensity of probe **9**-SPIN1 conjugate), which showed that the efficiency of the probe **10** with SPIN1 was 6% of that of probe **9** (in cell lysate). Similarly, the efficiency of probe **10** with KDM4A was 23% of that of probe **9** (in cell lysate). In revised manuscript, we have added the description of this section on Page 8, line 20.

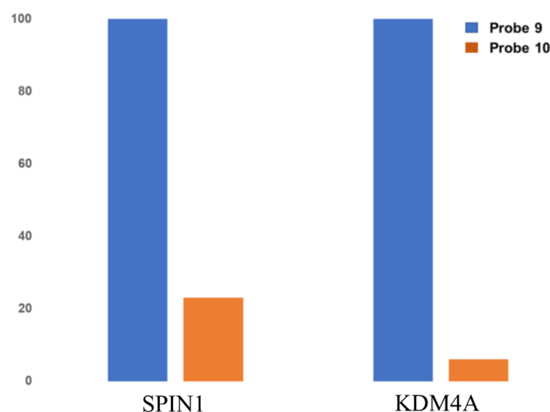

Fig. 3 Quantitation of the conjugation efficacy.

Furthermore, according to the suggestion of first reviewer, we employed LFQ proteomics to conduct a profiling of H3K4me3-interactors which significantly enriched 18 known H3K4me3-interactors in Hela cells using (FC > 1.5; P < 0.05) as cutoff. In contrast, using (FC < 0.67; P < 0.05) as a cutoff, we did not find any known H3K4me3 interactors. In conclusion, our new probe has good specificity with fewer problems of both false positives and false negatives. In revised manuscript, we have added the description of this section on Page 10, line 27.

2. You mentioned in your paper that 20 previously reported H3K4me3 readers were identified in your assay. Are these direct binders or the total number of proteins involving both direct and indirect binding proteins. Please elaborate on what percentage of known H3K4m3-binders were recovered in your assay.

Response: In revised work, we identified 18 H3K4me3-interactor, of which TAF2 (a component of the TFIID complex) and INO80B (a core component of the chromatin-remodeling INO80 complex) indirectly interact with H3K4me3 (11%), and the rest of the proteins interact with H3K4me3 directly (89%). In the revised manuscript, we have added the description of this section on Page 10, line 21.

3. Please include the confocal microscopy images of RAW264.7 cells treated with probes.

Response: As shown in Fig. 6b (in revised manuscript), CLSM analysis confirmed the successful delivery of these probes into RAW264.7 cells and localized in the nucleus.

4. Fig. 6. When comparing the results from Fig. 5 and Fig. 6, one would wonder why less proteins were identified using probes 12 and 13. Are there proteins overlapping between Fig. 5a and Fig. 6c?

Response: In this study, we perform the profiling of the interactors of histone lactylation by using our probe for the first time, and confirmed the interaction between AF9 and H3K9la by ITC (K_D=191 μM). Combined with ITC results, we consider that the weak interaction between AF9 and H3K9la may be the main reason for the low FC value. We further speculate that unlike the strong interaction of crotonylation with AF9, the epigenetic factor AF9 may be a "blunted recognition" reader of histone lactonylation.

Based on above results, we consider that the H3K9la modification may have fewer interacting proteins and weaker binding affinity compared to the H3K4me3 modification, which may result in the fact that fewer proteins were identified. Among the significantly enriched proteins (H3K9la FC > 1.2), three proteins (Ubtf, Rpl36a, Rpl18a) were identified in both Fig. 5a and Fig. 6c.

5. Putative histone lactylatio binding proteins were identified in this datasets. It would be ideal if the authors could validate additional proteins.

Responses: Thanks for your suggestions. We also analyzed other captured proteins and found that only four proteins had a higher FC value and a lower p value than AF9. Two of them are histones (H1-3, H1-5), one is a subunit of the large ribosome (Rpl36a), and another one is a thiol-specific peroxidase. The function of these proteins was not closely correlate with histone lactylation, so we did not perform the binding affinity tests.

6. Given the promising results, one would wonder if the presented approach could be used for quantitative studies, i.e. profiling differential hPTM binding proteins between distinct conditions.

Responses: Thanks for your suggestions. The H3K4me3 interactomes enriched by our probe were compared with two recent datasets obtained by using SILAC quantification proteomics. We found that SILAC quantification proteomics indeed has the advantage of reducing the rate of false positives (*Nat. Chem.* **2020**, *12*, 520-527; *Mol. Cell* **2021**, *81*, 2669-2681.e9). In the future study, we will utilize SILAC quantitative proteomics combined with our new probe to further explore the interactomes of histone PTMs *in situ*, and look forward to more in-depth results.

7. There are a few typos and grammatical errors in the manuscript.

Response: In revised manuscript, we have made corrections.

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Manuscript Title: Development of nucleus-targeted histone-tail-based photoaffinity probe to profile the epigenetic interactome in native cells

Corresponding author: Yi-Ming Li, Prof. Dr.; Lei Liu, Prof. Dr.

Reviewer 1:

In the revised manuscript, the authors have made commendable efforts to improve their proteomic data, including the use of LFQ to replace the previous TMT experiments and the addition of a proteomic study profiling H3K9cr binding proteins to showcase the generalizability of their strategy. Many of my previous concerns have been addressed. However, a few issues remain that I believe still need attention:

Response: We appreciate your positive comments.

1. Compared to the previously developed protein trans-splicing strategy that incorporates both the photo-affinity group and post-translational modifications (PTM) into native chromatin, the current probes are based on histone tail peptides. This approach cannot fully recapitulate the native chromatin environment, and this limitation should be discussed in the manuscript. Additionally, the term "histone photoaffinity probe" may be misleading, and I suggest using "histone-tail-based photoaffinity probe" to better reflect the nature of the probes.

Response: Thanks for your suggestions. We discussed the limitation of new probe in revised manuscript (Page 19, paragraph 2): Current peptide-based photoaffinity probes lack the context of the nucleosome or chromatin structure, which may cannot fully recapitulate the native chromatin environment, thus leading to some failure in identifying chromatin binders. We also used "histone-tail-based photoaffinity probe" to replace "histone photoaffinity probe".

2. LC-MS Data: I appreciate the authors' explanation for the additional peaks observed in their LC traces. However, some of the ESI-MS spectra still appear unclear. While I understand that certain peaks result from the loss of N_2 and the TFA adducts, there are additional unassigned peaks (notably in Figures S5C, S16C, S26B, and S27C) that resemble impurities. It would be beneficial if the authors could repurify these probes and provide cleaner spectra to improve the overall quality of the data.

Response: Thanks for your suggestions. We have repurified these probes and provide cleaner spectra.

Minor Issues:

• Figures 4d, 4e, and 5f: I recommend the authors double-check the references in these figures, as some are not found in the reference section.

Response: In the revised manuscript, we revised and added relevant literature.

Reviewer 2:

The authors have addressed most of the comments raised by this reviewer. They have provided a decent amount of data that support their conclusions, and their arguments are well taken. Overall, the quality of the manuscript has been improved, so this reviewer would suggest publication if they can address the following comment.

Response: Thanks for your positive comments.

Figure 6. Obviously AF9's binding affinity to H3K9la is lower than the other types of acylations, such as

acetylation. Even though ITC data in Figure 6c demonstrates this binding in vitro, more evidence is needed to consolidate it in cells. The authors are expected to provide more evidence to demonstrate that this finding is biologically relevant. For example, the authors could perform an IP experiment in Raw264.7 cells with or without sodium lactate treatment and compare the level of AF9 enrichment using confocal microscopy, WB, or mass spec.

Response: Thanks for your suggestions. To further investigate the binding of AF9 and H3K9la in vivo, we conducted a co-immunoprecipitation (co-IP) assay in Raw264.7 cells, both with or without sodium lactate treatment. We observed that upon stimulation with sodium lactate (50 mM), the levels of H3K9la and AF9 protein were increased compared to the control group, which did not receive sodium lactate treatment (Fig. 1a, input). Moreover, the co-IP results confirmed that sodium lactate treatment significantly enhanced the interaction between H3K9la and AF9 (Fig. 1a IP, 1b). All the results provide more evidence to demonstrate that this finding is biologically relevant. In revised manuscript, we have added the description of this section on Page 16, paragraph 2.

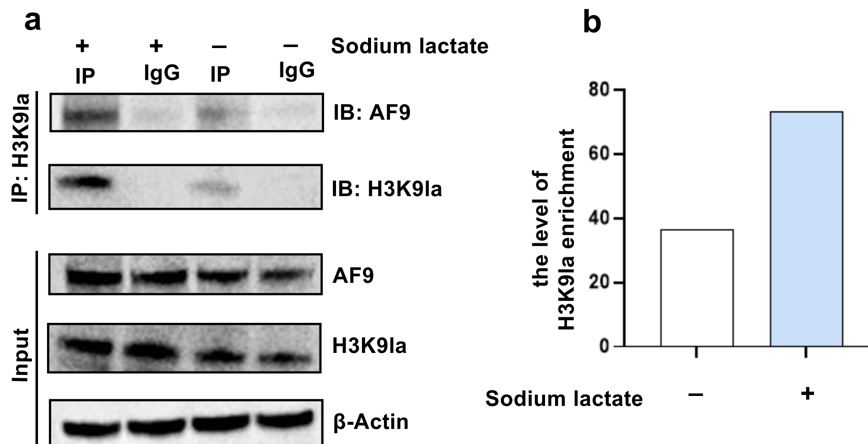

Figure 1a) CO-IP assay was used to detect the interaction of H3K9la and AF9 in RAW264.7 cells with or without sodium lactate treatment. b) Statistical quantification map of band grey values in result IB AF9.

We greatly thank these referees for the insightful suggestions that greatly help us improve the quality of the paper.

With my best wishes,

Yi-Ming Li, Ph.D.

Lei Liu, Ph.D.