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Development of a DNA-Templated Peptide Probe for Photoaffinity Labeling and Enrichment of the Histone Modification Reader Proteins

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Abstract: *Histone post-translational modifications (HPTMs) provide signal platforms to recruit proteins or protein complexes to regulate gene expression. Therefore, the identification of these recruited partners (readers) is essential to understand the underlying regulatory mechanisms. However, it is still a major challenge to profile these partners because their interactions with HPTMs are rather weak and highly dynamic. Herein we report the development of a HPTM dual probe based on DNA-templated technology and a photo-crosslinking method for the identification of HPTM readers. By using the trimethylation of histone H3 lysine 4, we demonstrated that this HPTM dual probe can be successfully utilized for labeling and enrichment of HPTM readers, as well as for the discovery of potential HPTM partners. This study describes the development of a new chemical proteomics tool for profiling HPTM readers and can be adapted for broad biomedical applications.*

Nucleosome, the fundamental packaging unit of DNA inside eukaryotic cells, is composed of a segment of DNA wrapped around a histone octamer, which itself consists of two copies of each of four core histone proteins (H2A, H2B, H3, and H4). A unique feature of histones is the presence of extensive post-translational modifications (PTMs), especially at their N-terminal tails. Histone PTMs (HPTMs), such as lysine acetylation, lysine methylation, and serine phosphorylation, have been considered to be a major type of epigenetic mark[1] as they are involved in almost all chromatin-related regulation events, including gene transcription, DNA replication, and chromatin remodeling.[2] In addition to extensive investigations into how "writer" and "eraser" enzymes regulate histones by adding or removing PTMs, special attention has also been focused on the binding partners, that is, "readers", of HPTMs, because the readers are recruited to recognize specific modifications and then to regulate distinct downstream biological outcomes. It has been shown that the disruption of this recognition and translation process contributes to the development of many human diseases, such as cancer and Alzheimer's disease.[3] Therefore, the identification of HPTM readers is critical to reveal the underlying regulatory mechanisms of many essential cellular events. Currently, multiple families of conserved protein domains have been identified that can specifically recognize HPTMs through a certain binding module.[4] However, compared with well-studied HPTMs,[5] large numbers of readers remain unidentified because of the absence of a reliable and sensitive method that can profile all HPTM readers.

Various screening methods, such as array-based screening[6] and biotinylated single peptide based immunoprecipitation,[7] have been developed to analyze the readers of HPTMs.[8] However, the noncovalent interactions between HPTMs and readers are rather weak, transient, and are highly dynamic, thus it is extremely challenging to use the aforementioned screening methods. To overcome these issues, a strategy using chemical probes containing photo-crosslinking groups in combination with mass-spectrometry-based proteomic profiling has been developed.[9] Using this approach, the probe can carry: 1) HPTMs of interest that can recruit specific readers, 2) photoreactive crosslinking groups that can convert noncovalent interactions into irreversible covalent bonds, and 3) a tag that can be used for detection or affinity purification of captured proteins. However, this chemoproteomics approach has suffered from complicated synthetic steps and the potential problem that the introduction of photoreactive groups into the middle site of the peptide skeleton may alter the peptide structure, which might further affect other features that are essential for the specific recognition of HPTMs by reader modules.

DNA-encoded chemical libraries (DEL) can be employed for the conjugation of chemical compounds or building blocks to short DNA fragments for chemical synthesis.[10] DEL can be used to introduce desired groups in a site-specific manner through the self-assembly of complementary double-stranded DNA and to prepare an extremely large number of compounds.[11] It has inspired many exciting applications in protein detection,[12] protein assembly,[13] and regulation of

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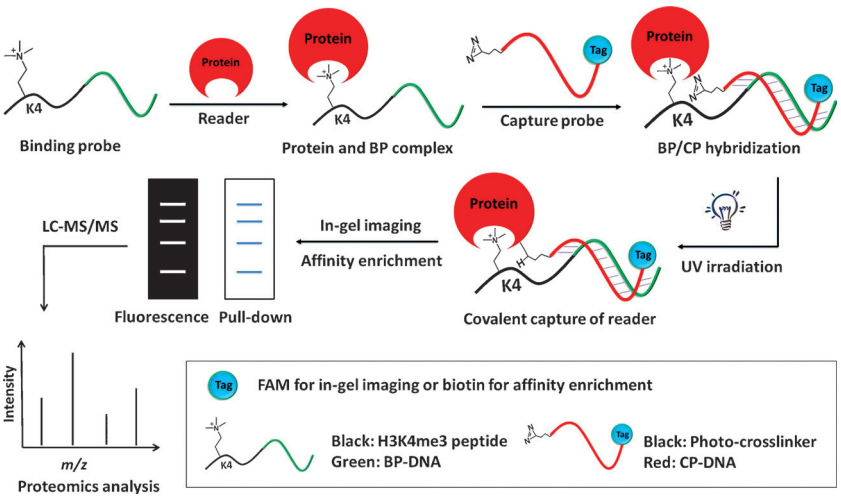
protein activity.[13b] In the present work, we combined DNA-templated technology with a photo-crosslinking method to design a HPTM dual probe as a novel HPTM peptide-based photoaffinity approach for the identification of histone readers. This probe provides the spatial flexibility necessary, through DNA-templated chemistry, to enable the photo-crosslinker to be close enough to the target proteins. This strategy enables us to covalently capture even low-affinity reader proteins by photo-crosslinking without affecting the binding efficiency between HPTMs and the readers.

A brief description of the strategy is shown in Scheme 1. Briefly, a "binding probe" (BP) was generated by conjugating a single-stranded DNA to a HPTM-carried peptide, while a "capture probe" (CP) was prepared by conjugating a photoreactive group and a tag used for detection or purification to both ends of a complementary single-stranded DNA, respectively. To this end, diazirine was chosen as the photoreactive group considering its smaller size, higher labeling efficiency, and better photostability compared with other photo-crosslinkers, such as benzophenone and arylazide.[14] This photoreactive group can create carbine or nitrene intermediates



Scheme 1. The preparation and application of the HPTM dual probe, based on DNA-templated chemistry and photo-crosslinking, for the identification of HPTM reader proteins.

under long-wavelength UV light ($\lambda = 330$ to 370 nm) which then proceed to form nonselective covalent bonds with the proximal amino acids of those bound proteins through addition reactions.[15] The conversion of noncovalent interactions into covalent bonds means that stringent washing conditions (such as the presence of a higher salt concentration and detergent) can be used to remove nonspecifically bound proteins. The synthesis and characterization of the probes are shown in the Supporting Information (Scheme S1, Scheme S2, and Figure S1).

The entire profiling procedure for the HPTM readers requires four main steps. Briefly, BP was first pre-incubated with protein samples to trap proteins that specifically recognize modified histones. Second, the addition of CP would initiate BP/CP hybridization that brings the diazirine groups close enough to the targets. Third, UV light was used to initiate the photo-crosslinking reaction. Finally, the captured proteins can be detected or affinity-purified by using FAM (5-carboxyfluorescein) or a biotin tag localized in the CP. For details, please see the experimental section in the Supporting Information.

The N-terminal tails of histones contain many evolutionarily conserved modifications, and histone H3 lysine 4 trimethylation (H3K4me3) is one of the well-characterized HPTMs. Accumulated evidence suggests that H3K4me3 plays an important role in transcriptional activation,[16] repression,[17] and recombination.[18] Therefore, we chose H3K4me3 as a representative to prepare probes in this study. As a well-known H3K4me3 reader, BPTF (bromodomain and PHD finger transcription factor) can bind HPTM through its PHD domain.[19] The binding strength of the BPTF–H3K4me3 interaction is roughly intermediate in value in terms of a variety of HPTM–reader interactions (Table S1). Here, the second PHD domain from BPTF (PHD_{BPTF}) was chosen as test substrate to examine the ability of the HPTM dual probe in the labeling and enrichment of reader proteins (the identification of the PHD domain is shown in Figure S2).

To optimize the experimental conditions aiming at a maximum yield of cross-linking products, the effects of different illumination times and salt concentrations on photo-crosslinking reactions were first investigated. As shown in Figure 1 a, the amount of photo-crosslinking products derived from bovine serum albumin (BSA) and CP-FAM (CP decorated with a fluorescent FAM group for detection) increases significantly with the extension of illumination time up to 6 min. An irradiation time greater than 6 min could not generate a higher yield of photo-crosslinking products. Therefore, a 6 min illumination time was used in all of the following experiments. Furthermore, a potential disadvantage of using diazirine is the appearance of nonspecific crosslinking products since its photoreactive groups lack specif-

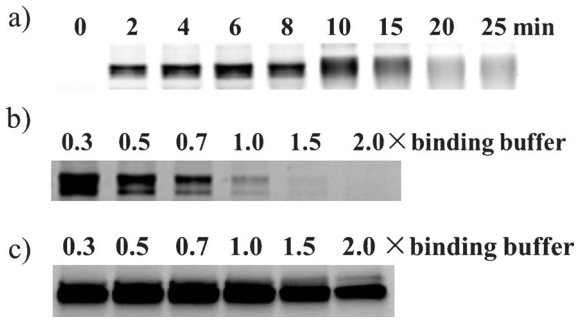


Figure 1. The optimization of labeling conditions. a) Analysis of the UV illumination time using BSA and CP-FAM. BSA = 10 μM, CP-FAM = 20 μM, $\lambda_{irrad} = 365$ nm . b) The effects of various concentrations of binding buffer on nonspecific binding. BSA = 15 μM, CP-FAM = 30 μM. c) The effects of various concentrations of binding buffer on specific binding. $PHD_{BPTF} = 8$ μM, BP/CP-FAM = 16 μM.

icity. Thus, we next sought to decrease nonspecific crosslinking by adjusting the salt concentration of the binding buffer because salt ions were shown to have an effect on both nonspecific and specific protein/DNA–protein interactions.[12c] As shown in Figure 1b, a remarkable decrease of nonspecific BSA-derived crosslinking products is detected with increasing salt concentration, in particular using the concentration 1× binding buffer (1× binding buffer components: 50 mM Tris-HCl, pH 7.8, 200 mM NaCl, 2.5 mM KCl, 2.5 mM $MgCl_2$, 1 mM $ZnCl_2$, 2 mM DTT, 0.005% NP-40; DTT = 1,4-dithiothreitol, NP-40 = nonyl phenoxypolyethoxylethanol. Note: the concentration 2× binding buffer means twice the concentration of all of the components of the 1× binding buffer). Moreover, the influence of salt concentration on specific binding was also investigated. As shown in Figure 1c, the yield of specific binding products decreases when the salt concentration is higher than that within the 1× binding buffer. Considering the effects of salt concentration on both nonspecific and specific binding, we finally chose 1× binding buffer as the reaction medium in all subsequent experiments.

To obtain the optimal spatial position of diazirine for an effective covalent binding of binders, the length of the CP can be varied to obtain better flexibility. Thus, we investigated the effect of the overhang length of CP ($n = 2, 4, 6, 8$) on the labeling yield. The n value indicates the number of overhang bases within CP that do not hybridize with BP after CP/BP hybridization ($n = 6$ is shown as an example in Figure 2a). As shown in Figure 2b, CP with a longer overhang gives better labeling yields, suggesting that a longer arm of CP indeed provides a better spatial position for the crosslinker to access binding proteins. However, a further increase to $n = 8$ did not significantly improve the yield of photo-crosslinking products, thus $n = 6$ was selected in our study.

Next, we designed a group of parallel in vitro experiments (Figure 3) to evaluate the labeling of the HPTM dual probe. PHD_{BPTF} was firstly incubated with BP and subsequently with

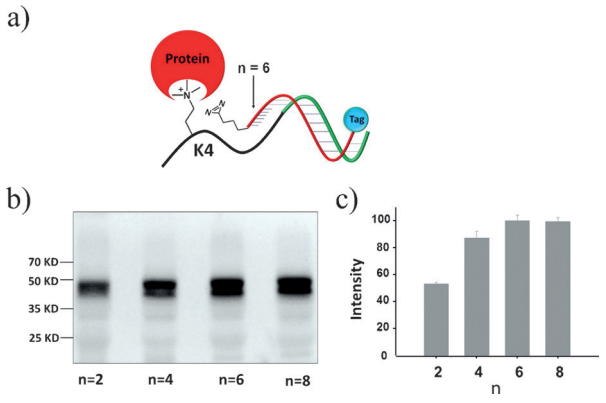


Figure 2. The optimization of the relative position of the photo-crosslinker towards the target by changing the overhang length within CP. a) An illustration of the $n = 6$ probe. b) SDS-PAGE analysis of parallel labeling experiments using CPs containing four different overhang lengths. $PHD_{BPTF} = 6$ μM, BP/CP-FAM = 12 μM. c) Relative fluorescence intensity of photo-crosslinking products versus n value. Error bars (based on standard deviation) derived from three independent experiments.

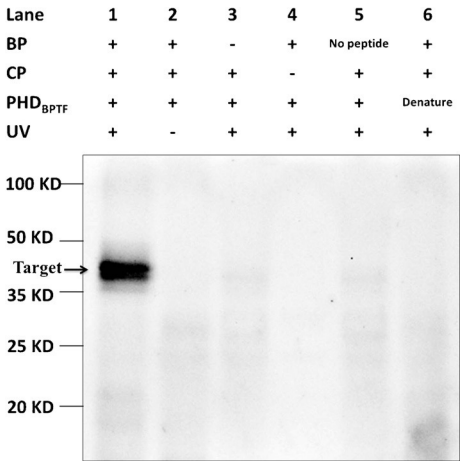


Figure 3. In vitro labeling of PHD_{BPTF} by the HPTM dual probe. $PHD_{BPTF} = 6$ μM, BP/CP-FAM = 12 μM. Lane 1: experiment performed in the presence of BP, CP, PHD_{BPTF}, and with UV irradiation. Lanes 2–6: negative control experiments for lane 1. Lane 2: no UV irradiation carried out. Lane 3: no BP. Lane 4: no CP. Lane 5: BP without H3K4me3 peptide. Lane 6: PHD_{BPTF} was heat-denatured.

CP-FAM followed by the photo-crosslinking reaction. As shown in Figure 3, the PHD_{BPTF} domain can be successfully labeled only when BP and CP are added and the sample is subjected to UV irradiation. There is no obvious PHD_{BPTF} labeling product in the negative controls (Figure 3, lanes 2–6: no UV irradiation, no BP, no CP, BP without modified peptide, denatured PHD_{BPTF}), indicating that all of the conditions, namely DNA hybridization, specific HPTM–reader binding, and adequate UV irradiation, are vital for successful protein labeling and that the observed labeling products are probe-specific rather than artifacts.

To explore whether the probe can be used to identify interactions between H3K4me3 and PHD_{BPTF} in a complex environment, we tested its selectivity in the presence of BSA. As shown in Figure 4a, the probe is able to robustly label PHD_{BPTF}. A similar labeling experiment of PHD_{BPTF} in a more complicated background, that is, the whole cell lysate, was also performed (Figure 4b), and again specific labeling was detected. Taken together, these results show that the HPTM dual probe shows an outstanding specificity and selectivity to its target even in a complex system.

We further characterized this HPTM dual probe for affinity enrichment from a complicated background using a biotin-decorated CP and a streptavidin beads system. As shown in Figure 5, the probe enables a specific enrichment of a protein matching to the expected molecular weight of PHD_{BPTF}, which was further confirmed by mass spectrometry after the protein band was excised from the SDS gel (Figure S3). Thus, it can be concluded that this HPTM dual probe is a valuable tool for the identification of HPTM readers when combined with a proteomics approach.

Finally, we investigated the application of this HPTM dual probe in a native environment by incubating the probe with the nuclear extracts of Hela cells to identify the endogenous interacting partners of H3K4me3. As shown in Figure 6a, there are visual protein bands shown in lane 4, indicating that

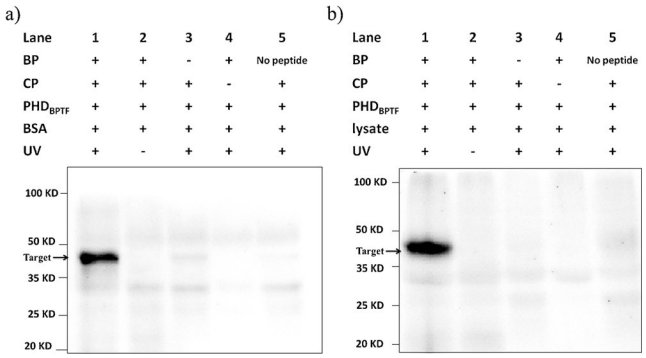


Figure 4. The selectivity of the HPTM dual probe for PHD_{BPTF} labelling in a) the presence of BSA (BSA = 6 μM, BP/CP-FAM = 12 μM, PHD_{BPTF} = 6 μM) and b) the whole cell lysate (PHD_{BPTF} = 6 μM, lysate = 50 μg per lane, BP/CP-FAM = 12 μM). The experiments were performed as in Figure 3 (lanes 1–5).

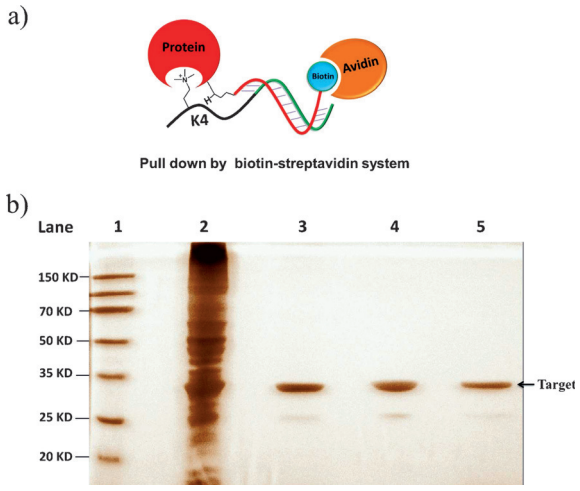


Figure 5. Affinity pull-down of PHD_{BPTF} by the BP/CP-biotin probe and streptavidin beads. a) The schematic diagram of affinity enrichment using the BP/CP-biotin and streptavidin system. b) Silver stain of proteins from the pull-down experiment. Lane 1: marker. Lane 2: PHD_{BPTF} mixed with Hela cell lysates and then loaded directly on the SDS gel. Lane 3: PHD_{BPTF} enriched from samples shown in lane 2 (PHD_{BPTF} = 6 μM, lysates = 50 μg, BP/CP-biotin = 12 μM). Lane 4: PHD_{BPTF} loaded directly on the SDS gel. Lane 5: PHD_{BPTF} after passing through the whole enrichment process loaded on the SDS gel (PHD_{BPTF} = 6 μM, BP/CP-biotin = 12 μM).

the probe gives a selective and sensitive enrichment of potential H3K4me3 binders. In contrast, no protein bands appear in the negative control of streptavidin beads (Figure 6a, lane 3), indicating that the enriched proteins are specific to the probe and not derived from nonspecific binding to agarose beads under the experimental conditions. Further analysis of the enriched proteins using HPLC-MS/MS (Figure 6a, lane 4) identified that the majority of the proteins contain PHD, Tudor, and WD40 domains (see Table S2 for molecular weights and a description of the proteins), which have been known to recognize H3K4me3 specifically. In the enriched group containing the PHD domain (Figure 6b), we identified some known readers of H3K4me3, such as BPTF, DIDO1, and PHF8.[20] Western-blot analysis further verified the enrichment of PHF8 (Figure 6c), which is usually difficult to detect because of its low abundance in native samples.[20b] Besides known readers of H3K4me3, we also identified many other proteins that have not been reported. For example, PARP1 that was identified in this analysis (Figure 6c) was reported in 2010 to prevent the demethylation of H3K4me3,[21] suggesting that it might be involved in the remodeling of chromatin structure and/or transcription regulation as a binder of H3K4me3. Together, these results demonstrate that the HPTM dual probe is highly promising in the screening of HPTM readers.

In summary, we have developed a HPTM dual probe for labeling and enrichment of histone readers by combining the photo-crosslinking method and DNA-templated chemistry. By using photo-crosslinking, this probe is able to convert those weak and transient intermolecular HPTM–reader interactions into covalent interactions, making further analysis much more convenient. While taking advantage of DNA-templated chemistry, it is possible to eliminate problems related to the internal localization of crosslinking groups affecting HPTM–reader recognition. Moreover, this probe mimics closely the status of HPTMs in native nucleosomes because of the presence of both HPTM and DNA within a single probe.[22] Using H3K4me3–PHD_{BPTF} as a representative model, we demonstrated that the HPTM dual probe selectively and sensitively enriches HPTM readers even in a native environment. By simply exchanging the peptide required in the probe skeleton, this HPTM dual probe approach holds great potential for a more broad and general

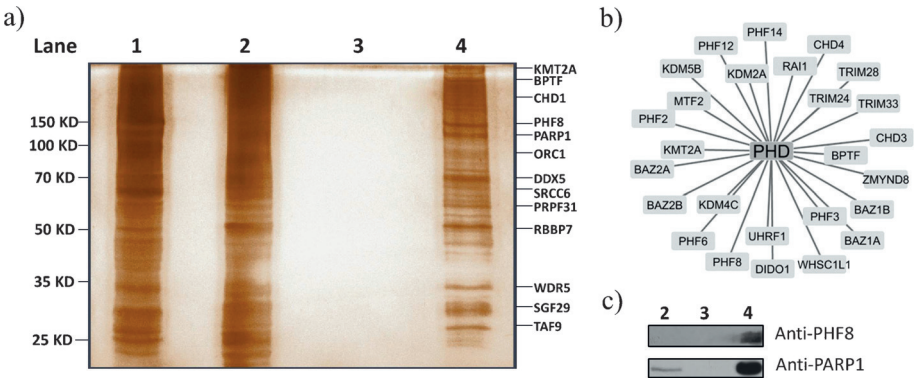


Figure 6. The HPTM dual probe selectively captures endogenous binders from nuclear extracts of Hela cells. a) Silver stain of the H3K4me3 pull-down experiment. Lane 1: nuclear extracts (10 μg loaded). Lane 2: supernatant after enrichment (10 μg loaded). Lane 3: streptavidin beads only as a control. Lane 4: binders isolated from nuclear extracts of Hela cells by HPTM dual probe and streptavidin beads. b) Proteins containing a PHD domain identified by mass spectrometry from lane 4 in Figure 6a (see Table S2 for details). c) After enrichment using the HPTM dual probe and streptavidin beads, the eluted proteins are detected by PHF8 and PARP1 antibodies.

application in the identification of other HPTM–reader interactions. Selective and sensitive enrichment methods for the identification of other such interactions are urgently required as a result of the many newly identified HPTMs[1b,23] and to gain a final complete understanding of the histone code.[2,4]

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