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RESEARCH ARTICLE

Identification of Histone-deacetylase (HDAC)-associated Proteins with DNA-Programmed Affinity Labeling

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Abstract: Histone deacetylase (HDAC) is a major class of deacetylation enzymes. Many HDACs exist in large protein complexes in cells and their functions strongly depend on the complex composition. The identification of HDAC-associated proteins is highly important in understanding their molecular mechanisms. Although affinity probes have been developed to study HDACs, they were mostly targeting the direct binder HDAC, while other proteins in the complex remain underexplored. We report a DNA-based affinity labeling method capable of presenting different probe configurations *without* the need for preparing multiple probes. Using one binding probe, 9 probe configurations were created to profile HDAC complexes. Notably, this method identified indirect HDAC binders that may be inaccessible to traditional affinity probes, and it also revealed new biological implications for HDAC-associated proteins. This study provided a simple and broadly applicable method for characterizing protein-protein interactions.

Introduction

Protein acetylation is an essential post-translational modification and histone deacetylase (HDAC) is a major class of enzymes regulating protein acetylation in cells.[1] Recent studies revealed that HDAC-mediated acetylome is very vast and far beyond histone modifications.[2] Aberrant HDAC activities are implicated in numerous diseases.[3] HDACs have been intensively pursued as drug targets and many HDAC inhibitors (HDACi's) have been developed to become clinical drugs.[4] Although the biological roles of HDACs are well recognized, considerable challenges still exist in studying their mechanisms. An important issue is that most HDACs exist in large protein complexes in cells.[5] Being in a complex modulates the deacetylation activity of HDACs, dictates their recruitment to specific genomic loci, and affects the interaction and crosstalk with other proteins.[6] Thus, efficient approaches capable of characterizing HDAC-associated proteins are highly desired. Previously, methods such as co-immunoprecipitation (co-IP) and affinity pulldown were used to study HDAC complexes. HDAC antibodies, peptides, HDACi's, and genetically tagged HDACs have all been used to isolate HDACs and the associated proteins.[5b, 6b, 7] Bantscheff, Schwarzer, and their respective co-workers demonstrated that small molecules are powerful tools to profile HDAC complexes.[6b, 7g, 8] Recently, photo-affinity labelling (PAL) probes have been developed to interrogate HDACs. PAL probes form covalent links with the target, so that the captured proteins could be reliably isolated with less interference from non-covalent interactions.[9] For example, Cravatt and co-workers designed a series of SAHA-BPyne probes to profile HDACs in cells.[9d, 9e] The Petukhov group developed the "BEProFL" and "photomate" probes to study HDACs.[9f-i] Recently, PAL probes were also used to study SIRT proteins[10] and other HDAC complexes.[9a-c, 11] However, affinity probes mostly capture the direct target, and the ability to capture indirect binders is rather limited. Moreover, affinity probe has a fixed structure; thus, the proteins it can capture are limited to the ones accessible upon target binding. Indeed, as seen in many studies, substantial synthesis efforts have been dedicated to prepare multiple probes with different configurations (varying the crosslinker, probe geometry, linker length and flexibility, etc.) to expand the target coverage.[7g, 9b-i, 12] For different analytical purposes, e.g. in-gel imaging or affinity purification, one would need to prepare multiple probes with different tags, which is a laborious and often highly challenging task.

Here we report a DNA-based affinity labelling method capable of presenting different probe configurations *without* the need to prepare multiple probes. We demonstrate the performance of this method by profiling HDAC-associated proteins with HeLa cells. Besides HDACs and known binders, many potential novel HDAC interactors were identified. Notably, this method identified a number of indirect HDAC binders that may not be accessible to traditional affinity probes and it also revealed new biological implications of HDAC-associated proteins.

Results and Discussion

Previously, we reported a DNA-based affinity labelling method named DPAL (DNA-Programmed Affinity Labelling; Figure 1a).[13] In DPAL, a ligand is conjugated with a DNA strand as the binding binding probe (BP) and a complementary DNA strand is

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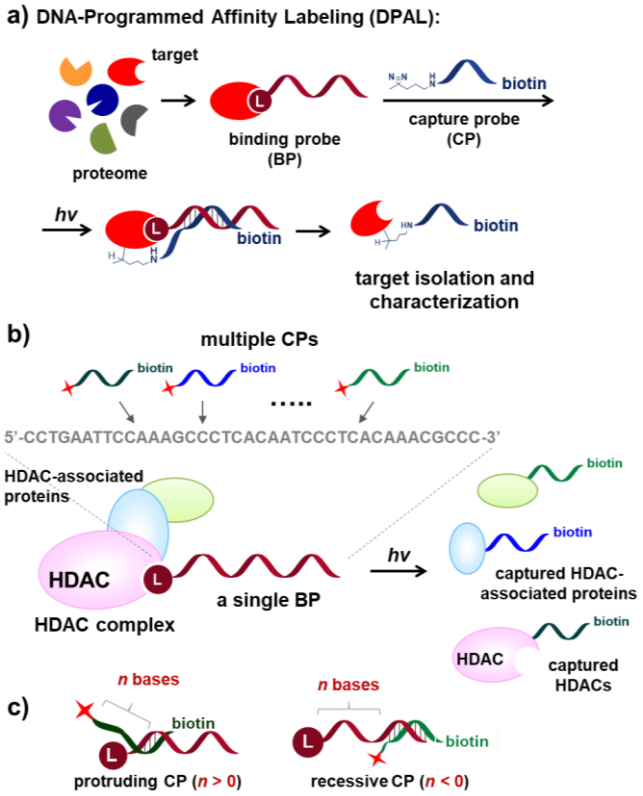

Figure 1. a) Scheme of DNA-Programmed Affinity Labelling (DPAL). b) Multiple CPs hybridize at different sites on BP for capturing the direct and indirect binders in protein complex. c) CPs may have a "protruding" ($n > 0$) or "recessive" ($n < 0$) configuration. L: a known ligand; star: photo-crosslinker.

conjugated with an affinity tag and a photo-crosslinker as the capture probe (CP). Upon target binding, BP hybridizes with CP and UV-irradiation triggers the capture of the target protein. DPAL has been used to identify the target of small molecules, peptides, nucleic acids, and aptamers.[13-14] With a similar concept, the Gothelf group developed an elegant method for site-specific antibody-DNA conjugation,[15] and the Tan group used aptamers to guide DNA-templated protein conjugation.[16] However, DPAL has not been exploited to study protein-protein interactions (PPIs). We reason that, instead of one BP/CP pair, hybridizing multiple CPs at different positions on the BP may capture not only the direct target, but also the proteins at different locations in the complex (Figure 1b). Changing the BP/CP hybridization position could flexibly vary the probe configuration without additional probe synthesis as only a single BP is required. Although multiple CPs are necessary, they are independent from the ligand and can be pre-prepared and used right off the shelf. As shown in Figure 1c, CP may be either "protruding" ($n > 0$) or "recessive" ($n < 0$); and CPs with large "n" values may capture the proteins distal to the ligand-binding site. We reason this approach may be suitable for characterizing HDAC-associated proteins, considering the multitude of diversity of HDAC complexes in cells.

First, we conjugated **SAHA** (suberoylanilide hydroxamic acid) to DNA as the BP (**BP-1**; Figure 2a). **SAHA** is a pan-HDAC inhibitor and can engage multiple HDACs;[8a] and the 4'-position of its phenyl ring could be modified without abolishing HDAC binding. A non-DNA affinity probe (**BP-2**) and a control probe (**BP-3**) without the critical hydroxamate, which chelates the Zn^{2+} in the catalytic pocket, were also prepared. First, the IC_{50} of **SAHA** and **BP-1** was determined to be 55 and 320 nM, respectively, suggesting that DNA conjugation lowered the activity but the probe remained active. **BP-2** showed an IC_{50} of 4.1 μM, possibly due to the branched probe shape.[9e] **BP-3** was inactive, precluding the possibility of inhibition by DNA. Next, **BP-1** was incubated with recombinant HDAC1 or HDAC3/NCoR2 complex and a series of biotinylated CPs, respectively (Figure 2c).[13b, 14b] All CPs contained a 15-nt region complementary to **BP-1** but with different spacers (n = +15 to -24). The mixtures were briefly irradiated (365 nm, 0 °C, 2 min.) and resolved with Western blot. As shown in Figure 2d-2e, most BP/CPs were able to label HDAC1, HDAC3, and NCoR2. Little labelling was observed with $n > +12$ or $n < -12$, while CPs with smaller n's (+6 to -6) had higher efficiency. Roughly the same amount of HDAC3 and NCoR2 were labelled, suggesting the crosslinker could access the protein beyond the **SAHA**-binding site. DNA-based probes may partially renature during electrophoresis, therefore resulting in a dual-band pattern.[13, 14b] The negative controls (**SAHA** competition, with **BP-3**, no UV) did not show significant labelling (Figure 2f). Furthermore, HeLa cell nuclear extracts were subjected to the same labelling procedure with **BP-1/CP** (n = +3), the biotinylated proteins were isolated with streptavidin beads and analysed with Western blot. As shown in Figure 2g, HDAC1, HDAC3, and the HDAC1/2-associated CoREST could be captured, and the capture was significantly reduced with **SAHA** competition. These results have shown that DNA-based probes were able to specifically label HDACs and HDAC-associated proteins.

Next, we performed more comprehensive profiling of HDAC-associated proteins with HeLa cells. First, **BP-1** was paired with a series of CPs (n = +15 to -12), respectively, to create 9 different probe configurations (Figure 3a). The BP/CPs were incubated with HeLa cell nuclear extracts and irradiated as same as in Figure 2. The biotinylated proteins were affinity-purified, during which strong washing conditions were applied to disrupt non-covalent interactions.[9d] The captured proteins were trypsinized and the resulting peptides were characterized with high-resolution liquid chromatography mass spectrometry (LC-MS/MS). The samples from **SAHA** competition were processed in parallel as control. For statistical robustness, all experiments were repeated for 3-5 times. The raw MS data was processed using the Perseus software to generate a volcano plot for each BP/CP (Figure S3). In each plot, the x-axis reflected the difference in protein abundance between the experiment and the control groups based on label free quantification (LFQ): the LFQ ratio (experiment:control) was calculated and binary logarithmized (log 2), and labeled as "log2 (fold change)". The y-axis was the minus common logarithm of the p-value of each data point and was labeled as "-log10 (p-value)" (see figure caption and the Supporting Information for more details on MS data processing). A two-sided Student's T-test was performed with a false discovery rate (FDR) = 0.05 and S_0 = 1 as the statistical threshold, shown as curved lines in each plot.[17] The proteins that met this threshold were considered as specific ones. The numbers of specific proteins are summarized in Figure 3b, and the complete protein list is provided in the Supporting Information.

The MS data showed the probes with large n's captured fewer proteins. Strikingly, at n = +3, the probes captured many more proteins, suggesting that a 3-base protruding spacer might be advantageous for the probe to access more proteins. A small number of overlapping proteins was observed (Figure 3b), indicating the spacer had significant impact on target coverage.

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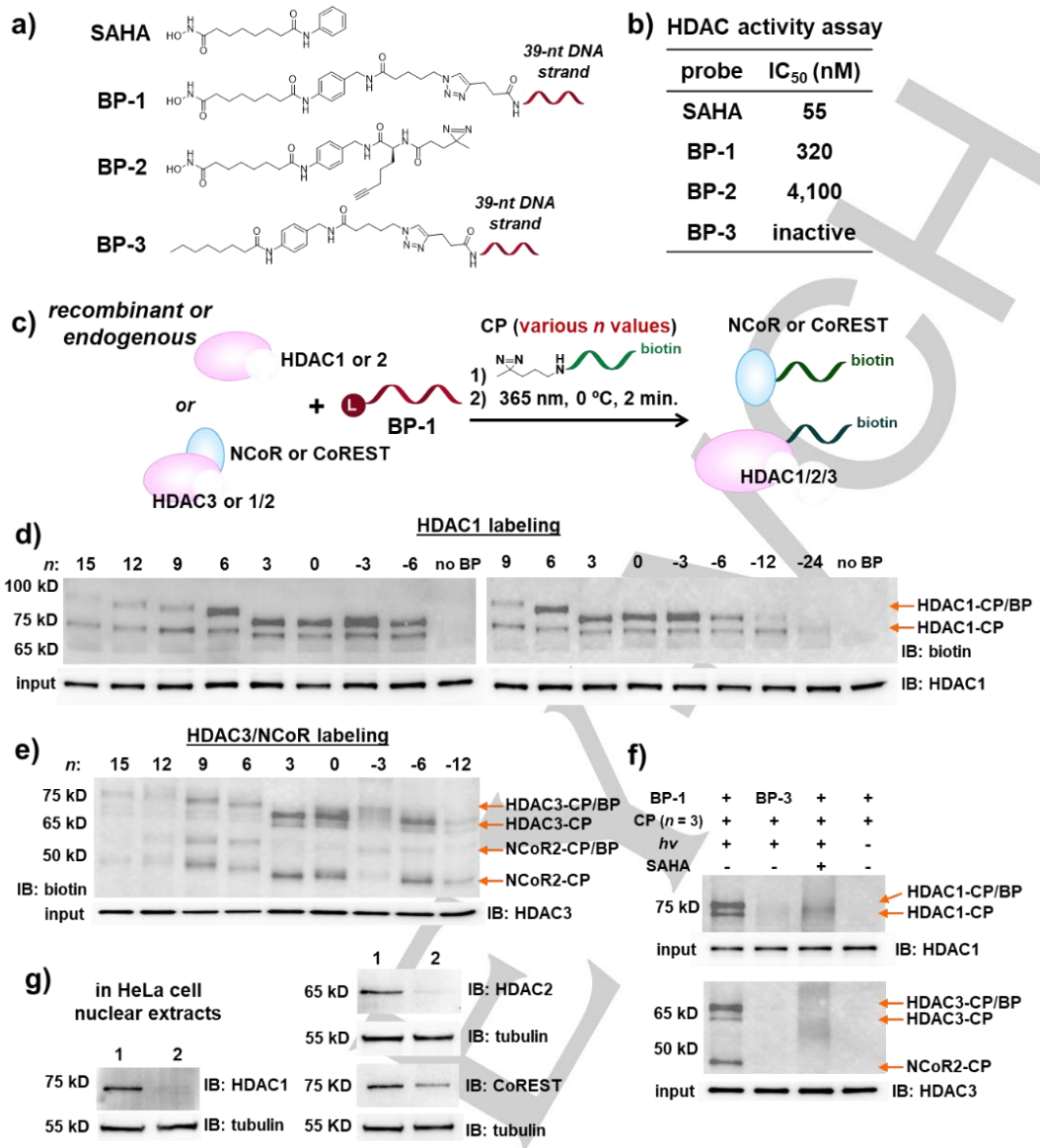

Figure 2. a) Structures of **SAHA, BP-1, BP-2**, and **BP-3**. b) HDAC activity assay results. c) **BP-1** and multiple CPs were used to label HDAC and HDAC complexes. Labeled proteins were analyzed with Western blot. Conditions: DNA, 1.0 μM; protein, 0.3 μM; 1x PBS (phosphate-buffered saline), pH 7.4, 0.1 M NaCl; UV: 365 nm, 0 °C, 2 min. L: **SAHA**. d)-e) Western blots of the labeled proteins; *n* values are as marked. f) Labeling specificity test. g) Labeling was conducted with **BP-1/CP** (*n* = +3) in HeLa nuclear extracts (2 μg/μL); biotinylated proteins were isolated with streptavidin beads and analyzed with Western blot. Lane 1: **BP-1/CP**; lane 2: with 20-fold excess **SAHA**. IB: immunoblotting. See the SI for details.

HDAC1, 2, 3, and 6 were detected (Table S1), which was consistent with the previous reports also using **SAHA**-based probes.[6b, 9a, 9d] HDAC3 exhibited very weak MS signal but the labelling could be verified with Western blot,[9a, 9d, 11] and we also observed similar phenomenon (Figure S4); the weak signal from HDAC6 may be due to its low abundance in nuclear extracts. Also similar to previous reports, Class-IIa HDACs were not identified, possibly because of its low affinity to hydroxamates.,[6b, 9a, 9c, 9d, 11] In addition, several known components of major corepressor HDAC complexes were identified (Table S1).

In total, 9 BP/CPs identified 267 unique proteins, which included HDACs, HDAC-associated proteins, and **SAHA**-binding proteins, such as the ones sharing the similar ligand space as HDAC or completely unrelated ones. To narrow down the list, the proteins were subjected to several filters (Figure 3d). First, we compared the list with the interactome of human HDAC1-11.[5b] Surprisingly, only 11 overlapping proteins was found, probably because of the different cell types and the probe structures. Next, the remaining 256 proteins was compared with the HDAC binders identified with small-molecule-based probes.[6b, 9a, 9d, 9e] Again, a small overlap of 16 proteins was observed, suggesting the DNA-based probes captured different sets of proteins from the small molecule probes. Furthermore, we conducted comprehensive literature survey and found that 128 proteins were either reported to interact with HDAC or annotated as deacetylase substrates based on acetylome analysis.[18] Finally, 112 proteins remained, which were considered as potential novel HDAC interactors, although it may still contain some **SAHA** binders.[9a] We tested the non-DNA probe **BP-2**;[9d] very few proteins were identified and only one passed the threshold (Figure S5), possibly due to the

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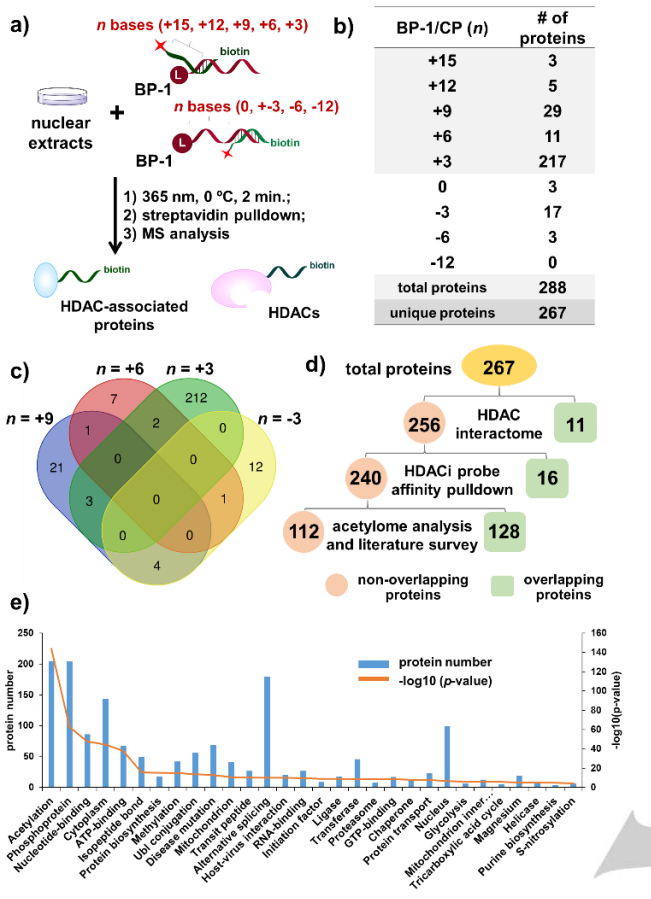

Figure 3. a) Protein profiling with 9 BP/CP configurations in HeLa cell lysates. Conditions were the same as Figure 2. b) Number of the specific proteins identified. c) Venn diagram showing the overlapping proteins for n = +9, +6, +3, and -3; others were not compared due to low protein number. d) Passing the proteins through three filters. e) Keywords analysis with DAVID functional annotation. Volcano plots are shown in Figure S3. Full protein list and experimental details are provided in the SI.

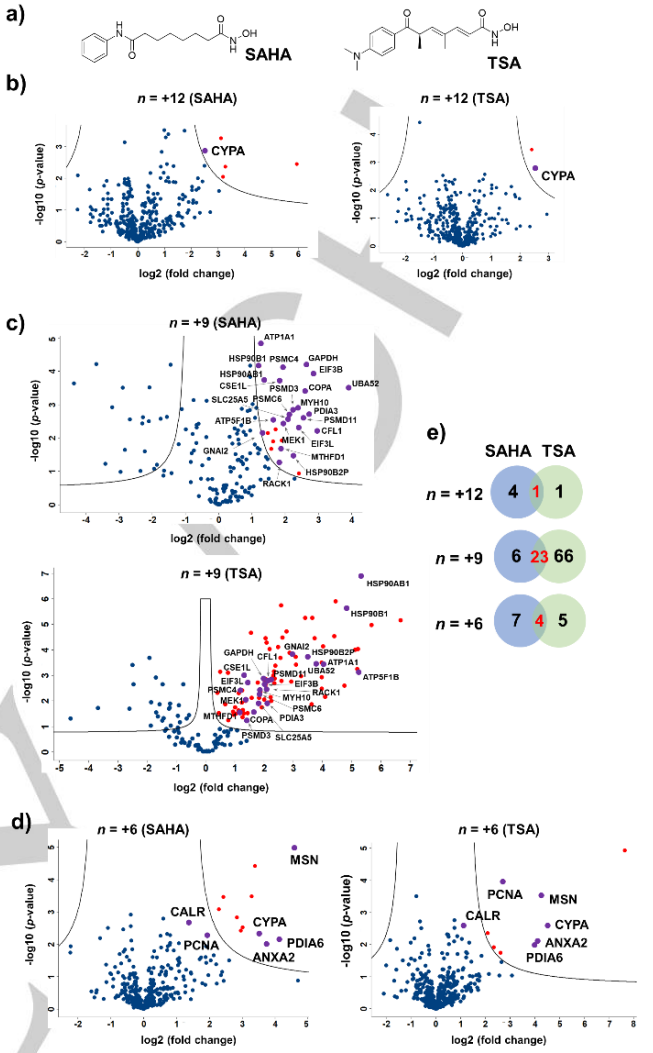

Figure 4. a) Structures of **SAHA** and **TSA**. b-d) Volcano plots (n = +12, +9, +6) from the **SAHA** and **TSA** experiments were compared. The curves denote the statistical threshold (two-sided Student's T-test; FDR < 5%, S_0 = 1). x-axis: log2 (fold change) = log2 (LFQ mean of the experiment group/LFQ mean of the control group); LFQ: label-free quantitation; y-axis: -log10 (p-value) of each data point. Red: proteins that passed the threshold; purple: overlapping proteins. e) Venn diagrams summarizing the identified proteins. See the Supporting Information for MS data processing and plotting details.

low binding affinity of **BP-2**. Finally, we performed keyword analysis using DAVID functional annotation (Figure 3e).[19] The top category is acetylation, covering 76.8% of the proteins with the most significant enrichment (p-value = 1.40 × 10^{-105}), which corroborated the profiling experiments where deacetylation complexes were targeted.

With this approach, the probes may possibly reach distal locations in the complex to capture indirect HDAC binders. Thus, we focused on the proteins identified with longer spaces (n = +12, +9, and +6). Since the proteins identified in Figure 3 may contain not only HDAC binders but also the ones that bind to **SAHA**,[9a] we performed an additional series of profiling experiments using trichostatin A (**TSA;** Figure 4a) as the competitor. **TSA** is also a pan-HDAC inhibitor;[20] thus, the proteins identified with both **SAHA** and **TSA** competitions are expected to be HDAC binders. The experiments using **TSA** were conducted the same as in Figure 3, and the volcano plots were shown side-by-side with the ones from the **SAHA** experiments (Figure 4b-d). The proteins that passed the threshold are highlighted in red and the overlapping ones are in purple.

At n = +12, cyclophilin A (CYPA), a known HDAC1/2 binder, was the only commonly identified protein (Figure 4b). At n = +9, **SAHA** and **TSA** identified many more proteins (Figure 4c). After passing through the filters, two potential novel binders were identified: MEK1 and COF1. MEK1 is a mitogen-activated protein kinase (MAPK) kinase. Interestingly, although many studies described the synergistic effects of MEK1 and HDAC inhibitors in anti-cancer therapy,[21] MEK1-HDAC binding was not reported. We performed co-IP in cell lysates using anti-HDAC1, 2, 3, 6 antibodies, respectively, which showed that MEK1 bound to HDAC1, but not the other HDACs; the reversed co-IP using anti-MEK1 antibody further confirmed the interaction (Figure 5a and S6a-d). Next, co-IP with recombinant HDAC1 and MEK1 confirmed that MEK1 directly binds to HDAC1. COF1 (cofilin-1) is an actin regulator in cells.[22] Co-IP screening with HDAC1/2/3/6 antibodies and reciprocal co-IP experiments showed that COF1 bound to HDAC1 in cell lysate but not between recombinant proteins (Figure 5b and S6e-i), suggesting that COF1 may bind HDAC1 indirectly through other proteins. At n = +6, four proteins were identified (Figure 4d), in which ANXA2 (annexin A2) was a potential novel binder.

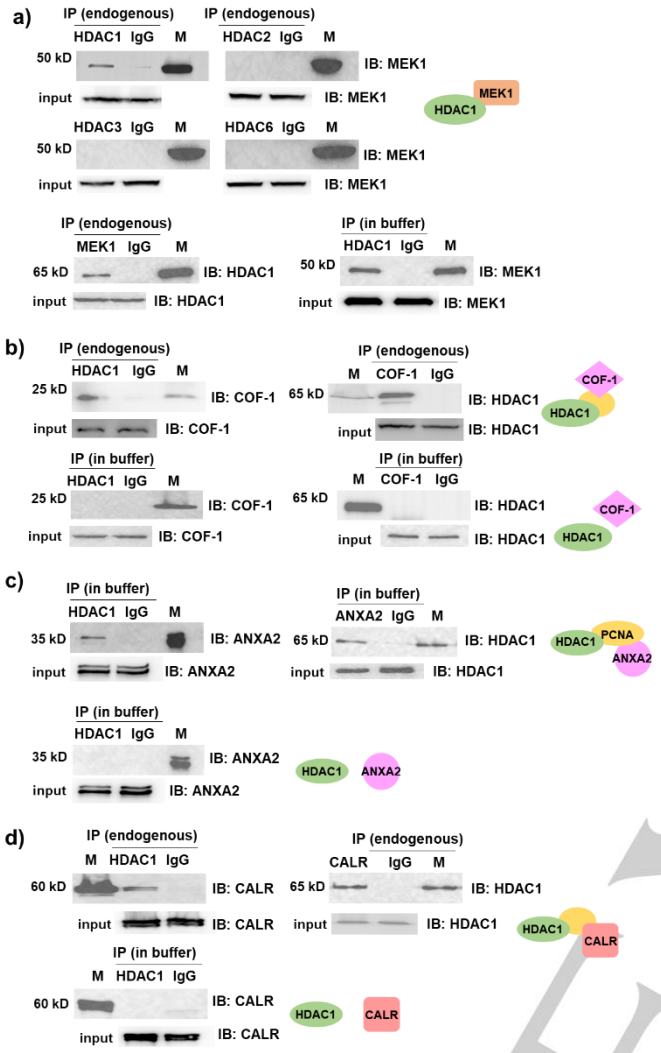

Figure 5. Co-IP experiments to validate the association of HDACs with selected proteins: a) HDAC/MEK1. b) HDAC/COF-1. c) HDAC1/ANXA2 with and without PCNA. d) HDAC1/CALR. M: pure protein or lysate, used as marker. IgG: Immunoglobulin G. Full gel images are shown in Figure S6.

Interestingly, PCNA (proliferating cell nuclear antigen), a known direct HDAC1 binder, was also identified (albeit slightly below the threshold); ANXA2 has been found to bind PCNA,[23] but its interaction with HDAC1 was not reported. Thus, we hypothesized that ANXA2 might bind HDAC1 indirectly through PCNA. As shown in Figure 5c and S6j-l, reciprocal co-IP using anti-HDAC1 and anti-ANXA2 antibodies confirmed the HDAC1-ANXA2 binding, while removing PCNA completely abolished the interaction. Moreover, co-IP with anti-HDAC2, 3, 6 antibodies did not show any interaction (Figure S6m). These results indicated that ANXA2 was an indirect HDAC1 binder and PCNA was required for binding. Lastly, we tested an additional protein CALR (calreticulin), which was only slightly below the threshold (Figure 4d). CALR is a chaperone protein participating in transcriptional regulation but not known to bind HDAC. Similarly, screening with anti-HDAC1/2/3/6 antibodies and reciprocal co-IP experiments showed that CALR was an indirect HDAC1 binder (Figure 5d and S6n-q).

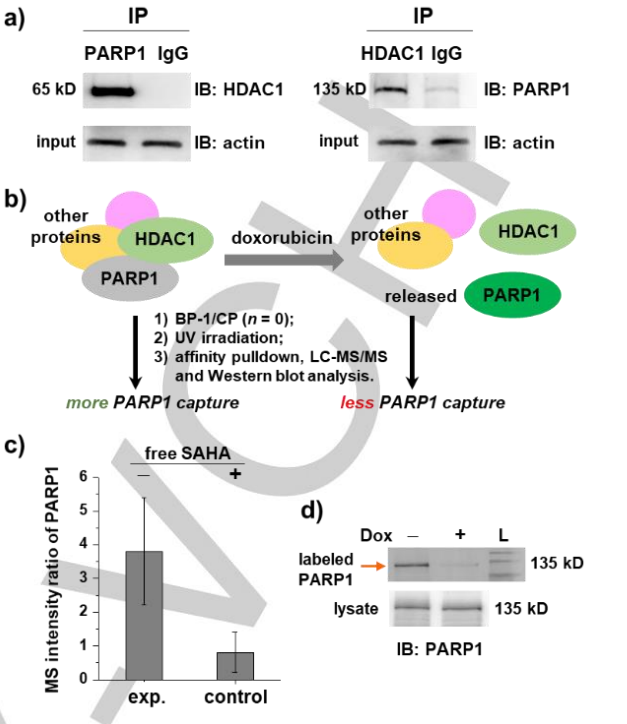

Figure 6. a) Reciprocal co-IP to validate HDAC1-PARP1 binding. b) Schematic representation of Dox-triggered PARP1 release. **BP-1/CP** was used to capture PARP1 in cells untreated or treated with Dox (0.5 µM, 16 hours). c) MS reporter ion intensities of PARP1 in b) were compared; exp.: without **SAHA**; control: with 20-fold excess **SAHA**. *y*-axis: MS intensity ratios = untreated cells/Dox-treated cells. Error bars represented three replicates. d) Top: **BP-1/CP** were used to affinity-purify PARP1 from HeLa cells; bottom: PARP1 expression level. Full gel images are shown in Figure S7.

Collectively, these results strongly suggested that COF1, ANXA2, and CALR bound to HDAC1 indirectly and demonstrated the method's capability in identifying indirect binders in HDAC complexes.

Furthermore, we employed this method to explore biological implications of HDAC-associated proteins. PARP1 (Poly[ADP-ribose] polymerase 1) is a nuclear protein regulating gene transcription. Although PARP1 is a known HDAC binder,[24] it had not been isolated by affinity probes. Here, PARP1 was captured by CP at n = 0; although below the statistical threshold, PARP1 showed low p-value (Figure S3) and high score/peptide coverage in MS data (Table S2). Reciprocal co-IP also verified the endogenous HDAC1-PARP1 interaction (Figure 6a). Under cellular stress, HDAC1-PARP1 binding would be disrupted, resulting in PARP1 release from HDAC1 complex (Figure 6b).[25] We investigated whether DPAL probes could detect such a process. HeLa cells were treated with the cytotoxic doxorubicin (Dox) to trigger PARP1 release,[25] then the nuclear extracts were labelled with **BP-1/CP**. The labelled proteins were affinity-purified and analysed with MS. By comparing the MS reporter ion intensities, significantly reduced PARP1 capture was observed in Dox-treated cells, while the control experiments with **SAHA** competition showed little difference (Figure 6c). Western blot also showed less PARP1 capture in Dox-treated cells, and Dox did not alter PARP1 expression in HeLa cells (Figure 6d). Although

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PARP1 may also interact with other HDACs, the dissociation from HDAC1 is a major reason for the decreased PARP1 capture.

Finally, we investigated whether the method could reveal novel cellular PPIs. PKM2 (pyruvate kinase isozymes M2) was identified at *n* = -3 (Figure 7a and S8). Previously, PKM2 was found to be associated with HDAC3 in glioblastoma and colon cancer cells;[26] however, the HDAC3-PKM2 interaction was highly dependent on cell stimulation. Upon EGF (epidermal growth factor) treatment, PKM2 formed a complex with HDAC3 to regulate histone H3 acetylation; without EGF, the HDAC3-PKM2 association was completely abolished (Figure 7b).[26a, 26b] Since the cells used here were not EGF-stimulated, we hypothesized that PKM2 might bind to other HDACs or bind to HDAC3 without the need for EGF-stimulation in HeLa cells. To investigate this, we first verified the status of EGFR signalling pathway. Serum-starved HeLa cells, either non-stimulated or EGF-stimulated,[27] were lysed, and then three protein phosphorylation markers in the EGFR pathway: EGFR/Y1068, Akt/S473, and ERK/T202/Y204,[28] were analysed with the respective assays (Abcam; EGFR: ab126438; Akt: ab253299; ERK: ab176660), which measured and compared the quantities of total protein and the phosphorylated protein. As shown in Figure S10, non-stimulated HeLa cells exhibited very low phosphorylation level for all three phosphorylation markers, while the total protein expression remained largely unaffected upon stimulation. These results indicated the EGF/EGFR pathway was suppressed in non-stimulated HeLa cells. Next, we performed co-IP of HDACs and PKM2 using anti-HDAC-1, 2, 3, 6 antibodies with the non-stimulated cells. Results showed that PKM2 could bind to HDAC1/3, but not HDAC2/6 (Figure 7c-f). To the best of our knowledge, it is the first time PKM2 is identified as an HDAC1-associated protein. Moreover, previous studies showed that HDAC3-PKM2 association deactivated HDAC3 and increased H3K9 acetylation in EGF-stimulated glioblastoma cells, while the effect was reversed in colon cancer cells (Figure 7b),[26] suggesting the function of HDAC3-PKM2 complex is highly context-dependent. We knocked-down PKM2 expression with small interfering RNA in non-stimulated HeLa cells and observed little change in the level of HDAC1, HDAC3, histone H3, and acetylated H3K9 (Figure 7g). Interestingly, the acetylation at H3K18, another major HDAC1/3 deacetylation site,[29] exhibited markedly decrease, suggesting PKM2 association might deactivated HDAC1/3, and the corresponding complexes might regulate histone H3 at different positions (e.g. H3K18) in HeLa cells. Also, studies showed EGF-stimulation triggered PKM2 translocation into nucleus.[26] In HeLa cells, little change of PKM2 in both cytoplasm and nucleus was observed upon EGF stimulation; the nucleus of non-stimulated cells appeared to already contain substantial amount of PKM2 (Figure S11). Although further studies are certainly needed, these results suggested that PKM2 in HeLa cells may function through a different mechanism. Collectively, using PARP1 and PKM2 as representatives, we have demonstrated that this method could reveal new biological implications for HDAC-associated proteins.

Conclusion

In summary, we have developed an affinity labelling approach for characterizing PPIs in protein complexes. Affinity probes are powerful tools to explore biology, but they are mostly designed to engage the direct target protein. Using affinity probes to

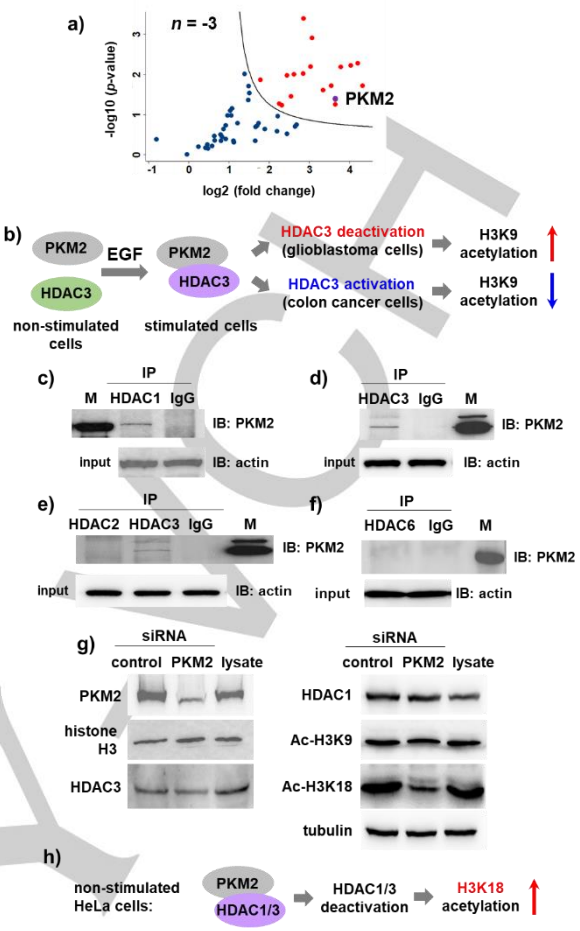

Figure 7. a) Volcano plot of the identified proteins at *n* = -3. The curves denote the statistical threshold (two-sided Student's T-test; FDR < 5%, S0 = 1; *x*-axis: log2 (fold change) = log2 (LFQ mean of the experiment group/LFQ mean of the control group); LFQ: label-free quantitation; *y*-axis: -log10 (*p*-value) of each data point. b) EGF stimulation is required for PKM2-HDAC3 binding, leading to either HDAC3 deactivation or activation HDAC3 in different cells.[26] c-f) Co-IP to examine PKM2-HDAC interactions in non-stimulated HeLa cells. M: cell lysate. g) Western blots to analyze the effects of PKM2 knockdown in HeLa cells; control: with scrambled siRNA; lysate: HeLa cell lysate. h) Results indicate that PKM2-HDAC1/3 interaction exists in HeLa cells without stimulation, which may deactivate HDAC1/3 and lead to increased H3K18 acetylation. Full gel images are provided in Figure S9. See the Supporting Information for details on MS data processing and plotting.

capture indirect binders in protein complexes remained underexplored. Here, using only one binding probe, 9 different probe configurations were created to profile HDAC complexes, and a large number of HDAC interactors were identified; notably, three of the four proteins tested (Figure 5) appeared to be indirect binders. This method is also responsive to cellular context change and could reveal new biological implications for HDAC-associated proteins. In addition, the probe system is modular and the guiding ligand could be easily changed to interrogate other protein complexes beyond HDACs.

This method has some limitations that may be further pursued. First, since DNA is membrane impermeable, the probes cannot be used in live cells, precluding the characterization of the PPIs that are disrupted during cell lysis. Encouragingly, the recent report on intracellular screening of DNA-encoded chemical library (DEL) showed cell-penetrating peptides could enable intracellular operation of DNA-conjugated small molecules.[30] Second, this

study used pan-HDAC inhibitors to engage multiple HDACs and identify more proteins, but additional steps were necessary to deconvolute the specific interactions. Using isoform-specific ligands would provide more specific information on individual targets. We will perform more in-depth studies and explore the biological applications of this method in future studies.

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Keywords: DNA-protein conjugation • DNA-templated synthesis • histone deacetylase • affinity probes • photo-affinity labelling

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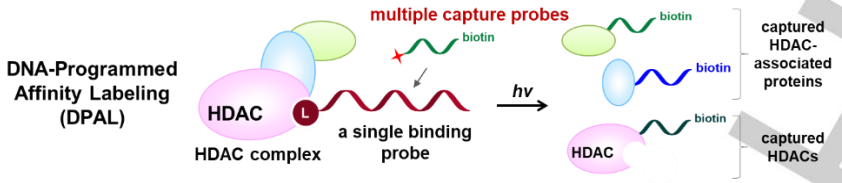

DNA-based affinity labeling probes enabled the capture and characterization of HDAC-associated proteins, including the direct and indirect binders in HDAC complexes. This method provides a simple and broadly applicable chemical tool to study protein-protein interactions.