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Fluorescent Probes for Single-Step Detection and Proteomic Profiling of HDACs

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KEYWORDS: Fluorescent probe, HDAC, histones, photo-crosslinking, proteomic profiling

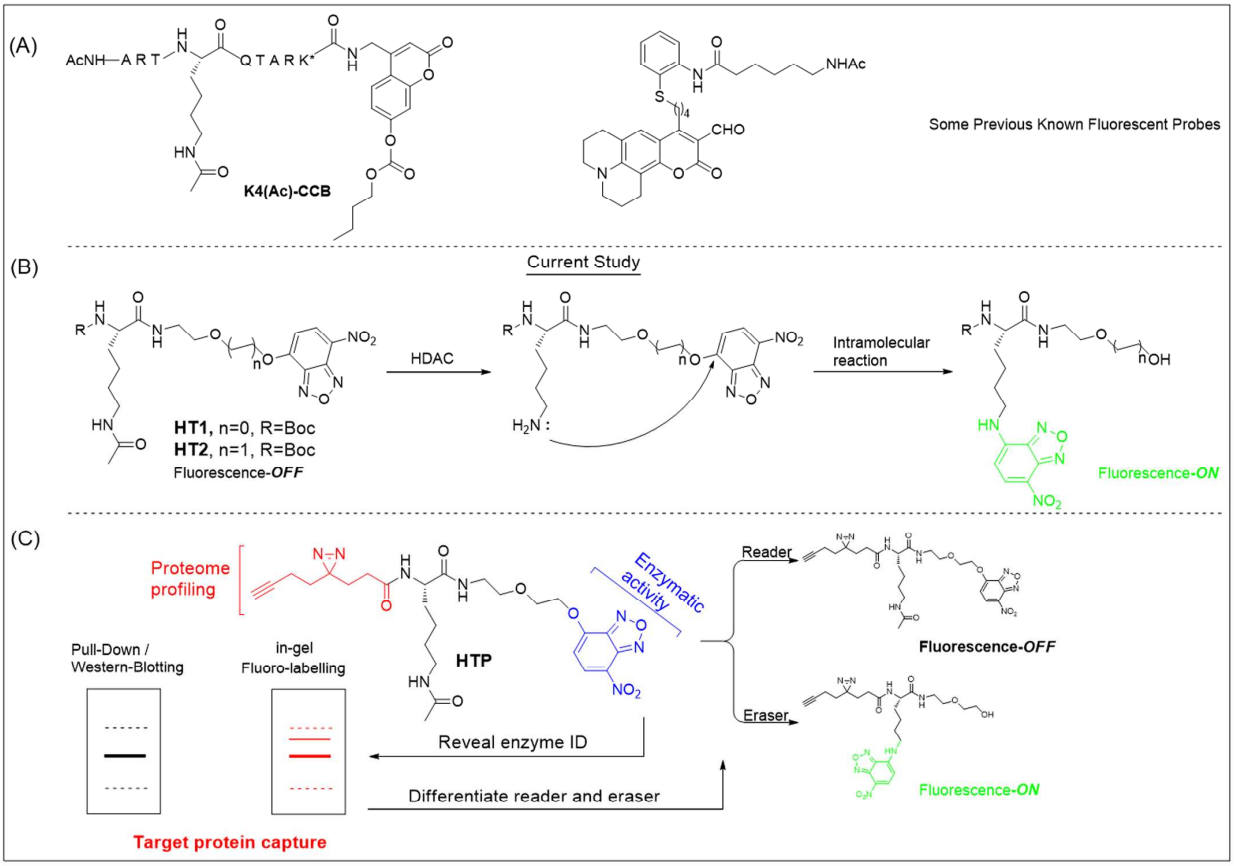
ABSTRACT: HDACs play important roles in regulating various physiological and pathological processes. Developing fluorescent probes capable of detecting HDAC activity can help further elucidate the roles of HDACs in biology. In this study, we first developed a set of activity-based fluorescent probes by incorporating Kac residue and *O*-NBD group. Upon enzymatic removal of the acetyl group in Kac residue, the released free amine reacted intramolecularly with the *O*-NBD moiety, resulting in turn-on of fluorescence. These designed probes are capable of detecting HDAC activity in a continuous fashion, thereby eliminating the extra step of fluorescence development. Remarkably, the amount of turn-on fluorescence can be as high as 50 fold, which is superior to the existing one-step HDAC fluorescent probes. Inhibition experiments further proved that the probes can serve as useful tools for screening HDAC inhibitors. Building on these results, we moved on and designed a dual-purpose fluorescent probe by introducing a diazirine photo-crosslinker into the probe. The resulting probe was not only capable of reporting enzymatic activity, but also able to directly identify and capture the protein targets from complex cellular environment. By combining fluorometric method and in-gel fluorescence scanning technique, we found that epigenetic readers and erasers can be readily identified and differentiated using a single probe. This is not achievable with traditional photoaffinity probes. In light of the prominent properties and the diverse functions of this newly developed probe, we envision that it can provide a robust tool for functional analysis of HDACs and facilitate future drug discovery in epigenetics.

Introduction

Acetylation of the ε-amino groups of lysine residues (Kac) is one of the most important histone posttranslational modifications (PTMs).[1] Kac modification can be recognized or deacetylated by epigenetic readers (e.g. bromodomains) and erasers (e.g. histone deacetylases, or HDACs) respectively. It has also been linked to many critical biological processes, including gene transcription, DNA replication and repair, etc.[2]

HDACs are hydrolases that catalyze the removal of acetyl groups from the ε-amino groups of lysine residues in histones or other cellular proteins.[3] To date, a total of 18 distinct HDAC proteins have been identified.[4] These proteins can be categorized into four classes according to their functions and structural similarities. Class I, II and IV belong to classical deacetylases, whose activity is zinc-dependent. Class III, on the other hand, consist of NAD^+-dependent proteins known as sirtuin 1 to 7 (SIRT1-7).[4a,5] They exhibit a different mode of action. Recent biological studies have revealed that sirtuins are involved in regulating various newly identified lysine PTMs such as ε-*N*-crotonyllysine (Kcr),[6] ε-*N*- succinyllysine (Ksuc) and ε-*N*-malonyl (Kmal).[7]

Numerous reports have shown that aberrant activity of HDACs is closely associated with the onset or progression



Scheme 1. (A) Molecular structures of some previously known HDAC probes.[21,22] B) Principle of our strategy to detect HDAC by fluorescent method. (C) Schematic illustration of the dual-purpose probe **HTP** used in the present study for detecting enzymatic activity and proteomic profiling studies.

of cancer and other diseases,[8] including Alzheimer's disease,[9] neurodegenerative disorders,[10] pulmonary disease[11] and asthma.[12] There have been long-standing interests in developing HDAC inhibitors as potential drugs, e.g. SAHA and FK228 for cancer treatment.[2b,8,13] Chemical tools that allow detection of HDAC activity can be highly useful for further advancement of HDAC research.

Over the years, a large number of chemical methods have been developed to detect HDAC activity, to facilitate functional annotation of HDACs or to help identify HDAC inhibitors. Most of these methods employ radioisotopes,[14] specific antibodies,[15] HPLC,[16] Mass Spectrometry[17] or electrochemistry.[18] Broad applications of these methods, however, have been hindered by laborious preparation work and multistep protocols. The more recent fluorescence-based methods have the advantage of high sensitivity and facileness.[8,19] One of the commercialized and commonly used fluorescence assays is based on substrate peptide coupled to fluorophore through a carboxy-terminal amide.[20] This method, however, has two major disadvantages. First, it is not a single-step process. An additional step of protease digestion has to be carried out. Second, the probe design is not flexible because the fluorophore needs to be conjugated to the carboxylate of the terminal lysine.

To address these problems, researchers have devoted a great amount of efforts in developing "one-step" fluorescent probes that are capable of detecting HDAC activity. The challenge of direct fluorescence method is that the aliphatic amide structure in the substrate does not allow π-conjugation of the group to a fluorophore and modulate the electronic state directly.[19] Only several notable one-step fluorescent probes have been reported so far.[21,22] The probe K_4(Ac)-CCB developed by Kikuchi's group is based on a fluorogenic coumarin to detect HDAC activity through an intramolcular transesterification reaction (Scheme 1A; left).[21] Another method developed by Buccella et al. employs intramolecular imine formation to turn on fluorescence (Scheme 1A; right) and monitor HDAC activity in real time.[22a] Very recently a supramolecular approach using a receptor probe was developed by Waters' group for detecting HDAC activity and screening inhibitors while this manuscript was in preparation,[22b] The previous two methods, however, have their own constraints. The probe in the first method, for example, is not very stable. Spontaneous hydrolysis of the carbonate moiety in the coumarin occurs after prolonged incubation

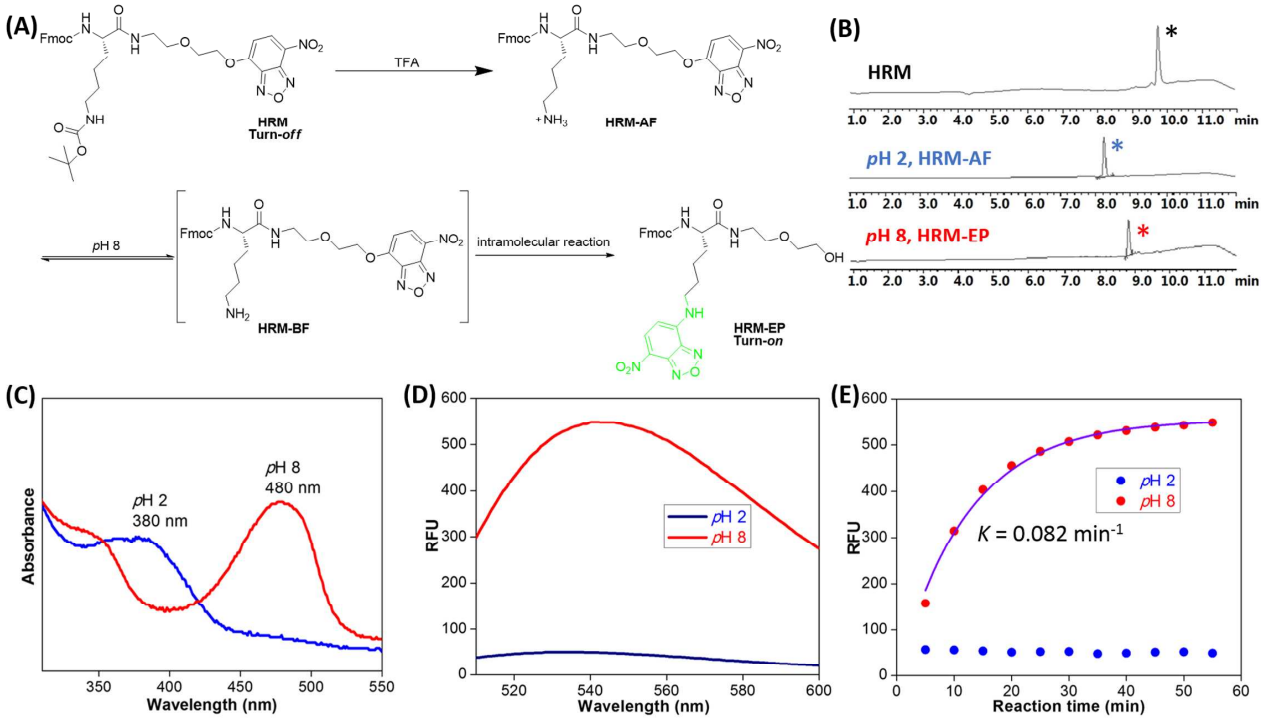


Figure 1. (A) Schematic illustration of the principle of **HRM** as a model probe. (B) Reversed-phase HPLC analysis of **HRM** (100 μM) at *p*H 2.0 and *p*H 8.0 after Boc-cleavage by TFA. The retention time of the peaks marked by asterisks were 9.8, 8.2 and 8.8 min respectively. (C) Absorption spectra of **HRM-AF** (100 μM) at *p*H = 2.0 and *p*H = 8.0. (D) Fluorescence spectra of **HRM-AF** (100 μM) at *p*H = 2.0 and *p*H = 8.0 (λ_{ex} = 480 nm). (E) Time-dependent fluorescence measurement of **HRM-AF** at *p*H = 2.0 and *p*H = 8.0 under described reaction conditions. The reactions were performed in duplicates.

without addition of enzyme. This results in some background fluorescence signal. In the second example, the fluorescence increase caused by imine formation is not significant, thus limiting the sensitivity of the probe. In light of these problems, it is highly desirable to develop novel fluorescent probes that can continuously monitor HDAC activity and perform drug candidate screening. It should also be noted that the fluorescent probe approaches based on HDAC substrates only provide information on the enzymatic activity of HDACs. They do not allow direct capture and identification of the associated enzymes from a complex cellular environment. The Identification of probe-interacting partners in cellular environment can provide useful insight into the regulatory mechanism of the proteins involved. However, the interaction between enzyme and probe is usually dynamic and transient, making it difficult for researchers to identify and capture the target enzyme in native conditions.[1b,23] On the basis of these factors, we expect that it is highly useful to develop new chemical tools capable of both detecting enzymatic activity and revealing the identities of the enzymes. Such tools can help further elucidate the roles of HDACs in epigenetic control and regulation.

In this study, we designed a panel of HDAC probes: **HT1**, **HT2** and **HTP** (Scheme 1B, C). **HT1** and **HT2** are one–step turn-on probes for monitoring HDAC activity in a continuous manner. Nitrobenzoxadiazole (NBD) is used because of its unique physical, chemical and fluorescence properties[24]: 1) *O*-NBD and *N*-NBD have distinctly different fluorescence properties; 2) *O*-NBD can be attacked by free amine to form *N*-NBD; 3) the relative small size of NBD group helps to minimize interference of the probe when binding to target enzymes. As shown in Scheme 1B, the *C*-terminus of *N*-acetyllysine (Kac) in our probes (**HT1** and **HT2**) is functionalized with an *O*-NBD fluorophore. In this configuration, the O-linkage of the NBD group results in a loss of fluorescence. Upon interaction with a suitable HDAC, enzyme-catalyzed deacetylation occurs on the probe. As a result, the free amine group in the lysine residue is released. The free amine undergoes spontaneous intramolecular exchange with *O*-NBD, leading to turn-on of fluorescence.

In the field of activity-based protein profiling (ABPP),[23] affinity-based probes (A*f*BPs) are often used as powerful tools for studying transient protein-ligand interactions.[25] This strategy utilizes photo-cross-linking to convert non-covalent protein-ligand interactions into irreversible chemical linkages. In our study, a **HTP** probe was designed both to detect HDAC activity and to conduct subsequent proteomic profiling studies (Scheme 1C). The probe consists of a Kac recognition unit, an *O*-NBD group and a minimalist photo-crosslinker. The photo-crosslinker contains an aliphatic diazirine group and a terminal alkyne group that are used in combination for downstream applications, e.g. in-gel fluorescence scanning and pull-down assays. We anticipated that **HTP**

could be used to 1) monitor the activity of HDACs in a continuous manner; 2) perform proteomic labeling and enrichment of targeted HDACs, so that target enzymes can be directly identified from complex cellular environments; 3) identify and differentiate epigenetic "readers" and "erasers"; 4) offer valuable insights to better understand the roles and the interactions of HDACs.

Results and Discussion

Model Studies with HRM. The probe **HRM** was designed to assess the feasibility and the efficiency of the intramolecular reaction between free amine and *O*-NBD under physiological conditions. In **HRM**, the deprotection reaction of the Boc group under acidic conditions acts as an enzyme-catalyzed deacetylation model of Kac, and an alkyl linker is incorporated between the free amine and *O*-NBD (Fig. 1A). When we designed **HRM,** we anticipated that intramolecular reaction might be preferred for 5-6 membered rings. For long-distance intramolecular reactions such as the case of **HRM**, the linker used should provide sufficient flexibility in order to overcome the ring strain. Therefore, we chose an aminoethoxyl linker in our design as the linker is flexible, causes less hindrance and can be further modified to different lengths. To test this hypothesis, the probe **HRM** was synthesized as shown in Scheme S1. Briefly, the carboxylic acid of Fmoc-K(Boc)-COOH was first activated with an NHS ester. The activated intermediate was subsequently treated with 2-(2-aminoethoxy) ethanol. The resulting compound was isolated and further reacted with NBD-F to obtain **HRM**. The product was confirmed by NMR and mass spectrometry respectively. Next, the Boc group in **HRM** was deprotected under acidic conditions to obtain **HRM-AF** (Fig. 1A). LC-MS showed that the Boc group was completely removed (Fig. 1B). A new peak with a retention time of 8.2 min appeared, and the mass of this new peak corresponded to the deprotected product (Fig. S1A). Subsequently TFA was removed. The residue was redissolved in PBS buffer, and the *p*H was adjusted carefully to 8.0. LC-MS analysis showed that another new peak with a retention time of 8.8 min was produced (Fig. 1B). This newly formed product also has the same mass value as **HRM-AF** (Fig. S1B), and it can emit strong fluorescence under UV light (Fig. S2). It has been previously reported that *N*-NBD-containing compounds are highly fluorescent whereas *O*-NBD-containing compounds are normally non-fluorescent or weakly fluorescent. From our observation in the **HRM** experiments, it can be concluded that **HRM-EP** was formed through a rapid intramolecular reaction between the free amine and *O*-NBD under buffer condition (Fig. 1A).

We next carried out detailed absorbance studies on **HRM-AF**. Spectrum analysis showed an absorption peak at 380 nm when *p*H = 2 (Fig. 1C). This absorbance wavelength also corresponded to that of the *O*-NBD group. When the *p*H was adjusted to 8.0, the absorbance peak quickly shifted to 480 nm (Fig. 1C), which corresponded to the absorbance peak of *N*-NBD. Subsequently, fluorescence studies were carried out. At *p*H = 2.0, the compound showed no fluorescence. However, when *p*H was adjusted to 8.0, a strong emission peak centered at 540 nm could be observed (Fig. 1D). Changes of fluorescence signals were also monitored in real time over the course of 60 min (Fig. 1E). Our results showed rapid increase of fluorescence over time and the reaction completed in about 30 min. The first-order rate constant k was subsequently determined to be 0.082 min^{-1} by fitting the fluorescence data to an exponential equation. In stark contrast, the fluorescence remained unchanged when *p*H was kept at 2.0. These results indicate that the intramolecular reaction of **HRM** can occur efficiently at *p*H = 8.0 when a significant amount of the deprotected amine in Kac exists in the neutral form (i.e. **HRM-BF**). To rule out the possibility that the fluorescence change of NBD is due to pH change, we synthesized two compounds NBD-N-Me and NBD-O-Me and tested their fluorescent properties in two different buffers (pH = 4 and 8). Results indicated that NBD-O-Me was non-fluorescent under both conditions, whereas NBD-N-Me showed high fluorescence and the intensity was almost not affected by the two pHs (Fig. S15). These results strongly prove that the fluorescence increment of HRM-AF is attributed to the intramolecular amine displacement.

Enzymatic Assay with Probe HT1 and HT2. After confirming the feasibility of intramolecular reaction in **HRM**, we moved further and designed two probes, **HT1** and **HT2**, which contain different linker lengths for HDAC detection. The synthetic routes of the two probes are similar to that of **HRM** (Scheme 2). Briefly, the carboxylic acid of Boc-K(Ac)-COOH was first activated with an NHS ester. The activated intermediate was then treated with 2-aminoethanol and 2-(2-aminoethoxy)ethanol respectively. The resulting compounds were reacted with NBD-F to afford **HT1** and **HT2**, which were characterized by NMR and Mass spectrometry.

To test the effect of linker length on the efficiency of both the enzymatic reaction and the subsequent intramolecular reactions, **HT1** and **HT2** were reacted with recombinant Sirt1 under identical conditions. Results showed that linker length had little influence on these tandem reactions (Fig. S3). **HT2** was thus chosen as the representative for subsequent studies. Absorbance studies with **HT2** showed a strong absorption peak at 380 nm in HEPES buffer. Upon addition of Sirt1 and NAD^+, a new absorbance peak appeared at 480 nm (Fig. 2A). In subsequent fluorescence experiments, **HT2** without enzyme or without NAD^+ displayed negligible fluorescence when excited at 480 nm (Fig. 2B). After Sirt1 and NAD^+ were added, however, a strong emission peak was observed at the same excitation wavelength. The fluorescence increase was determined to be as high as 50 fold. In addition, no fluorescence increase was detected when denatured Sirt1

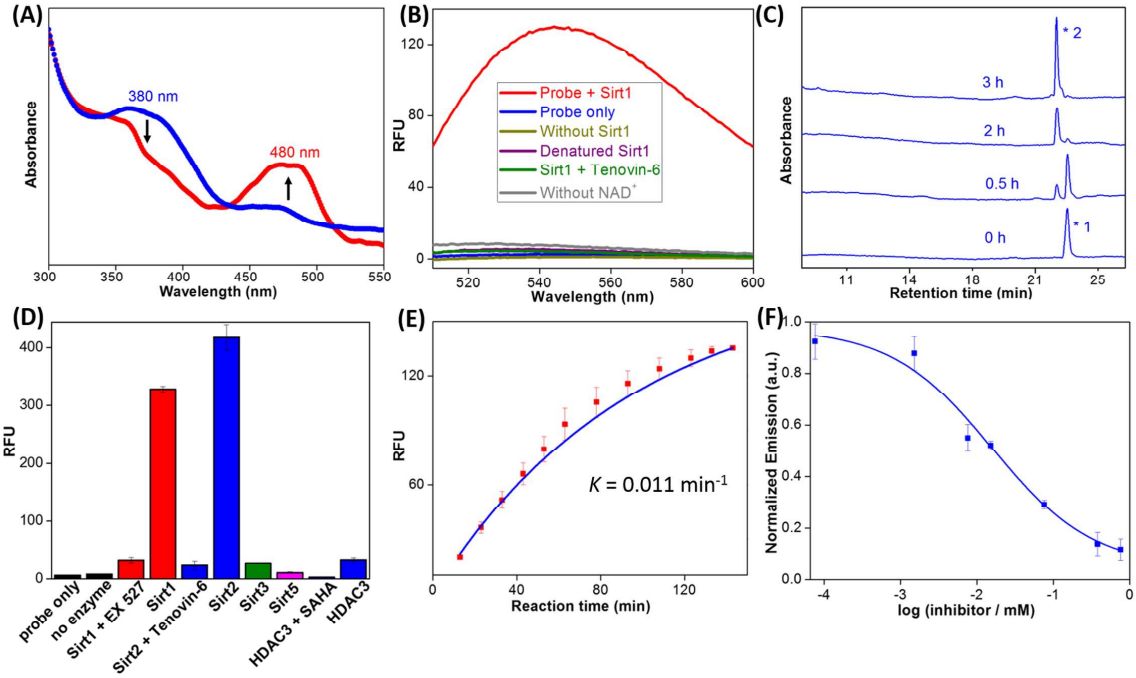
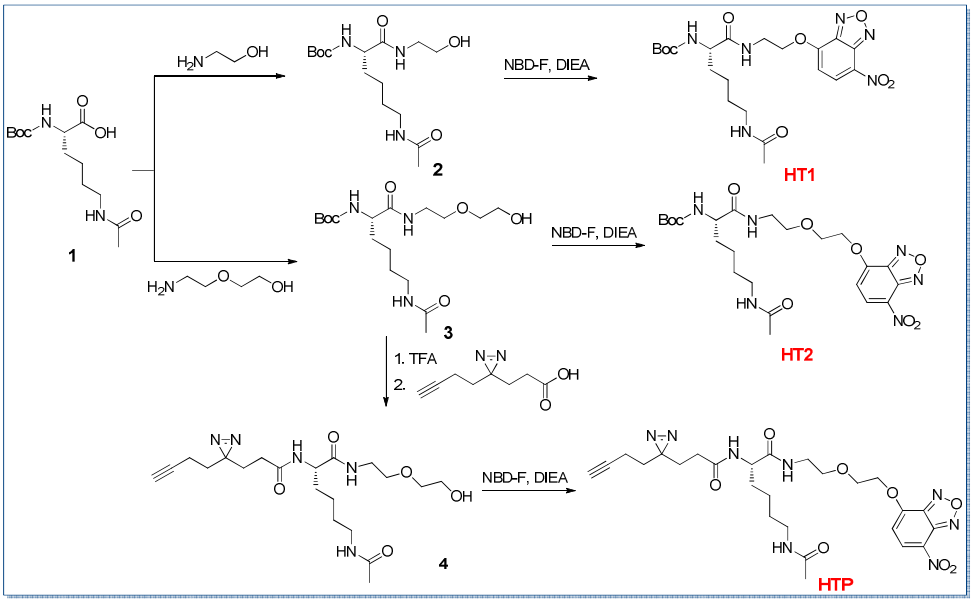


Figure 2. (A) Absorption spectra of **HT2** (10 μM) before and after enzymatic reaction with Sirt1 (0.1 μM) in 20 mM HEPES buffer (pH 8.0) containing 200 μM NAD at 37 °C for 2 h. (B) Fluorescence assay of **HT2** (10 μM) with Sirt1 (0.1 μM) under various conditions (λ_{ex} = 480 nm). (C) Representative HPLC analysis of the enzymatic reaction of **HT2** with Sirt2 (1 μM). The reaction was monitored at 254 nm. The retention time of the peaks marked with asterisk 1 and 2 was 22.8 and 22.2 min respectively. (D) Fluorescence detection of **HT2** (10 μM) with different HDACs (enzyme concentration: 0.1 μM, λ_{ex} = 480 nm; λ_{em} = 545 nm). (E) Time-dependent fluorescence measurements of **HT2** (10 μM) in the presence of Sirt1 (λ_{ex} = 480 nm; λ_{em} = 545 nm). (F) Dose-response inhibition curve of Sirt2 by Tenovin-6 using **HT2.** The reactions were performed in triplicates. Error bars were generated from the standard deviations of the three independent experiments.



Scheme 2. Synthetic routes for **HT1**, **HT2** and **HTP**.

was used or when an inhibitor was present. These experiments unambiguously confirm that the above reaction is indeed mediated by enzyme activity rather than other environmental factors.

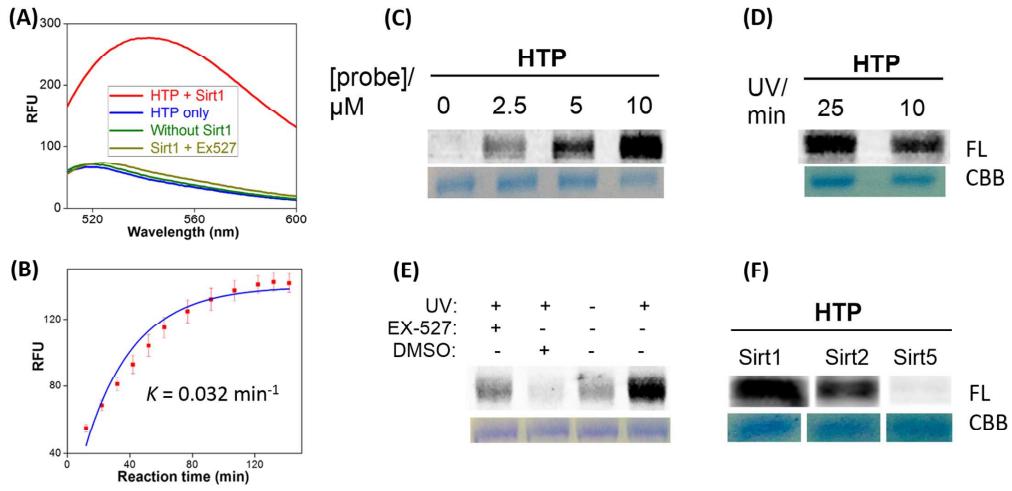


Figure 3. (A) Fluorescence spectra of the probe **HTP** (10 μM) measured in the presence and absence of various factors (λ_{ex} = 480 nm) for Sirt1. (B) Time-dependent fluorescence measurements of **HTP** (10 μM) in the presence of Sirt1. (C) Concentration-dependent labeling of **HTP** with recombinant Sirt1 (0.1 μg/lane) after 25-min of UV irradiation. (D) UV irradiation time-dependent labeling of **HTP** (5 μM) with recombinant Sirt1 (0.1 μg/lane). (E) Labeling of **HTP** (5 μM) with recombinant Sirt1 in the presence and absence of inhibitor (EX-527, 400 μM), 0.1 μg/lane. (F) Labeling profiles of **HTP** with Sirt1, Sirt2 and Sirt5. 0.1 μg/lane. CBB, Coomassie gel; FL, in-gel fluorescence scanning. The reactions were performed in triplicates. For (B-F), λ_{ex} = 532 nm.

Furthermore, we performed HPLC analysis to monitor the reaction (Fig. 2C). 40 μM **HT2** was incubated with Sirt2 for different periods of time at 37 °C in HEPES buffer (*p*H 8.0). HPLC analysis showed the appearance of a new peak after 0.5 h with a retention time of 22.2 min. The peak represented the reaction product. The **HT2** peak (retention time = 22.8 min) disappeared almost completely after 2.5 h. Further MS analysis showed the reaction product corresponded to the expected tandem deacetylated/exchanged product (Fig. S5B). Additionally, the collected fraction of this reaction product (at 22.2 min) showed strong green fluorescence under UV light (Fig. S6). HPLC experiments with Sirt1 under identical experimental conditions were carried out, and similar results were obtained (Fig. S5A).

Next we moved on to study the response of **HT2** with a series of other HDACs, including Sirt3, Sirt5 and HDAC3/NCOR1 (Fig. 2D & S8). Our results indicated that the probe displayed good activity towards Sirt1 and Sirt2. On the other hand, when Sirt3 was present, the fluorescence increase of the probe was not high, indicating that the probe might not serve as a good substrate for Sirt3. HDAC3/NCOR1 showed activity towards HT2, although much lower than that of Sirt1 and Sirt2. Sirt5 did not show any activity towards **HT2**, which is in good agreement with reported literature that Sirt5 recognizes Ksuc and Kmal preferably.[7b] We further conducted time-dependent enzymatic reaction with Sirt1 (Fig. S5C) and Sirt2 (Fig. 2E). The fluorescence intensity was weak initially. It increased rapidly during incubation and plateaued after 2-3 h. By fitting the fluorescence data to an exponential equation, the first order rate constant *k* of Sirt 1 and Sirt2 is determined to be 0.011 min^{-1} and 0.008 min^{-1} respectively. Concentration-dependent experiment of HT2 with different amounts of Sirt2 showed a good linear relationship between the reaction velocity and the enzyme concentration (Fig. S9), confirming that our assay can accurately measure HDAC activity. Furthermore, the Michaelis constant K_M of HT2 with Sirt2 was determined to be 58 μM using fluorescence method, which is in good agreement with HPLC measured value of 63 μM (Fig. S10). These experiments together prove that our probe can serve as a robust tool for detecting HDAC activity.

We also examined the suitability of our probe to assess the inhibition potency of Sirtuin inhibitors. A known inhibitor, Tenovin-6, was used in our study. Different concentrations of Tenovin-6 was incubated with Sirt2 for 30 min. **HT2** was then added and incubated for a further 2 h. Subsequently the fluorescence was measured, and the data was fitted into the equation to derive the EC_{50} value (0.016 mM, Fig. 2F), which turned out to be similar to the value reported in the literature (0.010 mM).[26] These data clearly prove that our probe can serve as a useful tool to monitor the enzymatic activity and screen potential inhibitors of HDACs.

Synthesis of Probe HTP and Enzymatic Activity Assay

The aforementioned studies prove that our first generation probes are suitable for studying enzymatic activity and screening inhibitors. Encouraged by these results, we next synthesized a dual-purpose probe **HTP** by introducing a photo-crosslinker diazirine into **HT2**. The probe was designed to enable proteomic studies in order for us to gain a deeper understanding of the enzymatic activity, the

enzyme abundance and protein/protein interactions of HDACs under native cellular environments. The synthetic scheme of **HTP** is shown in Scheme 2. Photo-crosslinker was synthesized as previously described.[25d] The key step involved the transformation of ketone to diazirine group. Compound **3** was synthesized as earlier mentioned. Subsequent TFA deprotection and coupling with the photo-crosslinker gave compound **4**. The NBD moiety was then installed to afford the final product, **HTP**, which was characterized by NMR and mass spectrometry. With **HTP** in hand, we first studied its enzyme-catalyzed activity. When Sirt1 and Sirt2 were added in the presence of cofactor NAD^+, a significant fluorescence increase was readily observed (Fig. 3A & S8A). No fluorescence increase was observed when the enzyme was absent or when an inhibitor was present. Time-dependent experiment showed that the enzymatic reaction of **HTP** with Sirt1 plateaued within 2 h (Fig. 3B). The first order rate constant *k* of Sirt 1 and Sirt2 was subsequently determined to be 0.032 min^{-1} and 0.015 min^{-1} respectively. These results indicate that **HTP** can be used to directly report the activity of HDACs in a standard fluorescence-based enzymatic assay.

In-gel Fluorescence Scanning Next, we assessed the ability of **HTP** to label recombinant enzymes through in-gel fluorescence technique. Sirt1 was chosen in our model study. Different concentrations (0, 2.5, 5 and 10 μM) of **HTP** was incubated with Sirt1 for 30 min in HEPES buffer and then irradiated with UV for 25 min. The samples were subsequently subjected to click reaction with Rhodamine-azide and separated by SDS-PAGE. In-gel fluorescence scanning was then conducted. As shown in Fig. 3C, a strongly fluorescent band was readily observed when the concentration of the probe was 2.5 μM. No fluorescence signal was observed when the probe was not added. The fluorescence intensity of the protein band increased with increasing concentrations of the probe. These results showed that the probe can effectively label the target protein in a concentration-dependent manner. Time-dependent experiments showed the labeling was quite intense when the sample was irradiated for 10 min (Fig. 3D). The fluorescence intensity of the protein band further increased when the irradiation time was prolonged, e.g. to 25 min. For inhibition studies, two inhibitors, Ex-527 and Tenovin-6, were evaluated. Both inhibitors showed competition effects in the labeling experiment (Fig. 3E and S12C). Concentration-dependent inhibition studies with Ex527 demonstrated that the inhibition is dose-dependent (Fig. S13). We also carried out labeling experiments with various HDACs, including Sirt1, Sirt2 and Sirt5. Results indicated that Sirt1 and Sirt2 could be strongly labeled, whereas Sirt5 did not show obvious labeling (Fig. 3F). These results indicate that the probe is highly selective towards Sirtuins that recognized Kac residue. On the other hand, the probe is not able to bind to their active site of Sirtuins that can not recognize Kac residue preferably, e.g. Sirt5. Hence, no labeling was observed in our experiments.

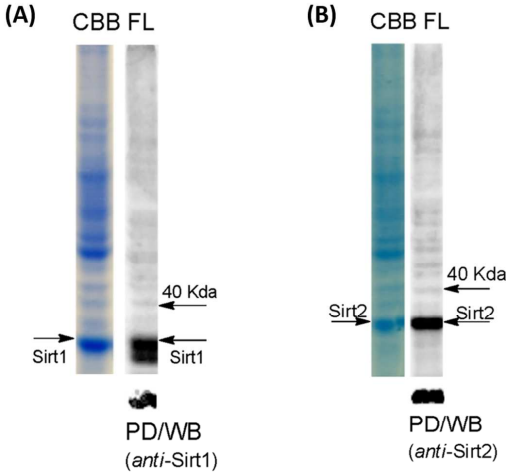


Figure 4. (A) Labeling of Sirt1-spiked (0.6 μg) HeLa lysate (15 μg) by **HTP** (10 μM), and the corresponding pull-down (PD)/ western blotting (WB) results (bottom). (B) Labeling of Sirt2-spiked (0.6 μg) HeLa lysate (15 μg) by **HTP** (10 μM), and the corresponding pull-down (PD)/western blotting (WB) result (bottom). CBB, Coomassie gel; FL, in-gel fluorescence scanning. The results were from two independent experiments. λ_{ex} = 532 nm.

Proteomic Labeling and Pull-Down Studies. Inspired by these results, we moved on to assess the ability of **HTP** to label Sirt1 and Sirt2 in a complex proteome environment. HeLa lysates were spiked with Sirt1/Sirt2, and then treated with **HTP**. The samples were subjected to UV irradiation. Click reaction and in-gel fluorescence scanning were subsequently carried out. As shown in Fig 4A & B, clear fluorescent bands, which correspond to the molecular weight of Sirt1 and Sirt2, were readily detected. This occurred despite the presence of a large excess of other cellular proteins, indicating that our probe is capable of labeling its target proteins from complex cellular lysates. We also carried out pull-down experiments to examine whether the fluorescent bands captured by the probe are indeed Sirt1 and Sirt2 present in complex cellular lysates. We incubated **HTP** with HeLa lysates spiked with 4% Sirt1/Sirt2. The samples were subsequently UV-irradiated and subjected to click reaction with biotin-azide. They were then incubated with streptavidin beads. The bead-bound proteins were analyzed by western blot with the corresponding antibodies (i.e. *anti*-Sirt1 and *anti*-Sirt2; Fig. 4). Results show that both proteins were successfully enriched under these conditions. We also investigated whether HTP probe can pull down the endogenous Sirt1 in native environment by incubating the probe with whole cell extracts of HEK 293 cells. A very faint band could be observed in the western blot experiments, which might be due to: 1) the endogenous Sirt1 is relatively low in the whole cell extracts under native conditions; 2) the binding affinity of HTP toward Sirt 1 is modest.

We then moved on to investigate whether our probe can be used to identify and differentiate epigenetic readers and erasers. The traditional affinity-based probes (A*f*BPs) based on Kac-containing peptide can be used to identify

both epigenetic readers and erasers,[1] but they are unable to differentiate these two classes of proteins. In contrast, **HTP**, besides its proteome-profiling capability, possesses the additional property to specifically detect the enzymatic activity of targeted HDACs due to its dual-purpose design. Therefore, we expected that **HTP** might be useful in differentiating between epigenetic readers and erasers. To test this hypothesis, we carried out in-gel fluorescence scanning experiments with BRD4-1 (reader) and Sirt2 (eraser). Both proteins showed strong labeling, indicating that **HTP** can indeed be used to identify them (Fig. 5B). In the enzymatic fluorescence assay, only the epigenetic eraser Sirt2 induced a significant fluorescence increase (Fig. 5A). These results together confirm that **HTP** can be used to differentiate these two important classes of epigenetic proteins.

To further explore whether this differentiating property can be applied in a complex cellular environment, we performed in-gel fluorescence scanning and enzymatic fluorescence assay with **HTP** by spiking Sirt2 and BRD4-1 respectively in mammalian total cell lysates. Results showed that both proteins can be identified in complex cellular environments (Fig. 4B & Fig. 5D). However, only Sirt2 spiked cellular lysate can induce a large fluorescence increase (Fig. 5C). These results indicate that **HTP** can serve

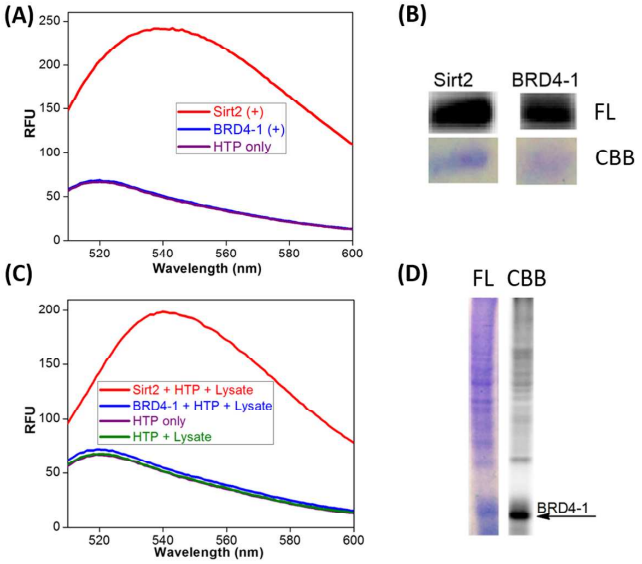


Figure 5. **HTP** was used to distinguish epigenetic reader and eraser. (A) Fluorescence spectra of **HTP** (10 μM) with Sirt2 and BRD4-1 (1 μM), 37 °C. (B) Labeling of **HTP** (10 μM) with recombinant Sirt2 and BRD4-1, 0.3 μg/lane. (C) Fluorescence spectra of probe (10 μM) with Sirt2 (0.4 μg) and BRD4-1(0.4 μg) in lysate (10 μg). (D) Labeling of BRD4-1-spiked HeLa lysate (15 μg/lane) by **HTP** (10 μM). CBB, Coomassie gel; FL, in-gel fluorescence scanning. The results were from two independent experiments. (A) & (C) λ_{ex} = 480 nm, NBD fluorescence, (B) & (D), λ_{ex} = 532 nm, Rhodamine fluorescence.

as a functional tool for differentiating epigenetic readers and erasers. It is therefore useful for elucidating the different roles of the two classes of proteins in epigenetic regulation. In addition, considering that some of the HDAC isoforms can still bind to the ligand while they are catalytically inactive, we expect that **HTP** can provide a new and simple tool for identifying functionally active epigenetic erasers for proteomic profiling studies.

Conclusion

In summary, we have developed a panel of novel fluorescent probes for HDACs. Among them, **HT1** and **HT2** are capable of monitoring HDAC activity in a continuous manner. The fluorescence increment can be up to 50 fold, which is better than the existing one-step HDAC fluorescent probes. More importantly, the probe **HTP** possesses multiple functions and can be used to 1) report the enzymatic activity of HDACs; 2) conduct proteomic profiling in complex cellular environment; 3) identify epigenetic readers and erasers and differentiate between them. We envision that the probe will serve as useful tools in proteomic study by identifying functionally active epigenetic erasers rather than binders. We expect that the sensitivity and selectivity of our probe may be further increased by installing selected peptide fragments into the probe. Although in this research work, the focus has been on sirtuins that recognize Kac residue, the study can be extended to other HDACs that can recognize the emerging new PTMs, including Ksuc, Kcr and others. We are currently working on new probe designs that can help to further facilitate research on HDACs.

Materials and Methods

General Information. Starting materials and solvents were purchased from commercial vendors and used without further purification, unless indicated otherwise. The required anhydrous solvents were produced according to common procedures. The required anhydrous conditions were carried out under nitrogen atmosphere using oven-dried glassware. TLC for Reaction monitoring was performed with pre-coated silica plates (Merck 60 F254 nm, 250 μm thickness), and spots were visualized by UV, ninhydrin, KMnO4 and phosphomolybdic acid or other stains. Flash column chromatography was carried out with silica gel (Merck 60 F254 nm, 70-200 mesh). ^{1}H-NMR, ^{13}C-NMR and ^{19}F were recorded on Bruker 300 MHz/400 MHz/500 MHz NMR spectrometers. The spectra were referenced against the NMR solvent peaks ($CDCl_3$ = 7.26 ppm, CD_3OD = 3.31 ppm, CD_3CN = 1.94 ppm) and reported as follows: ^{1}H: br (broad singlet), s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), dd (doublet of doublets). ^{13}C: chemical shift δ (ppm). Mass spectra were obtained on Shimadzu IT-TOF-MS or ABI Qstar Elite Q-TOF or PC Sciex API 150 EX ESI-mass spectrometers.

Analytical HPLC was carried out on a Shimadzu LCMS (IT-TOF) system with a reverse-phase Phenomenex Luna

5 μm C18 (2) 100 Å 50 × 3.0 mm column at a flow rate of 0.6 ml/min. Water and acetonitrile were used as eluents. UV absorption spectra were obtained on a Shimadzu 1700 UV/Vis Spectrometer. Fluorescence measurement was recorded with a FluoroMax-4 fluorescence photometer. *p*H value was recorded with a FiveEasy TM Fe20 *p*H meter. In-gel fluorescence scanning of the SDS-PAGE gels was carried out with a FLA-9000 Fujifilm system. Western blotting was carried out with a C600 Azure biosystem. HDAC3/NCOR1 was purchased from Enzo Lifescience. Recombinant sirt1 was purchased from Abcam. Sirt1 and Sirt2 were recombinantly expressed and purified as previously described.[7b] Sirt5 was a gift from Cheryl Arrowsmith (Addgene plasmid # 25487). Sirt3 was a gift from John Denu (Addgene plasmid # 13736). Sirtuin proteins and BRD4-1 were expressed and purified according to the reported procedure.[27,28] Antibodies of Sirt1 and Sirt2 were purchased from Cell Signaling. Secondary antibody (IRDye 680RD Donkey anti-Rabbit IgG) was purchased from LI-COR Biosciences. Immobilon-FL poly(vinylidene difluoride) membrane was purchased from Merck Millipore. Streptavidin Magnetic Beads were purchased from New England Biolabs.

Enzymatic Reaction. The enzymatic activity of Human sirtuins were measured by detecting the fluorescence signals with a fluorescence photometer. The probe was incubated with sirtuins at 37 °C for 2.5 h in the presence of NAD in 20 mM HEPES buffer (*p*H 8.0) containing 150 mM NaCl, 2,7 mM KCl and 1 mM $MgCl_2$. The first-order rate constant, k, was calculated by fitting the fluorescence data shown in Figure 1E, 2E, 3B, S5C, S8B to Equation:

Fluorescence intensity = 1 – exp (-kt).

Cell Culture. HeLa cells were cultured in Dulbecco's modified Eagle medium (1X, DMEM) containing 100 units/mL penicillin, 100 μg/mL streptomycin, 10% heat-inactivated fetal bovine serum (FBS; Invitrogen) and sodium pyruvate (1mM) (Thermo Scientific). The mixture was maintained in a humidified 37 °C incubator with 5% CO_2.

Preparation of Cellular Lysates. Protein lysates were prepared with the following procedures: cells were washed twice with cold phosphate-buffered saline (PBS). Lysis buffer (RIPA buffer) was then added, and the cells were harvested with a cell scraper and transferred to 1.5 ml EP tube. Subsequently the cells were lysed with gentle shaking for 30 min, and sonicated in an ice bath. Finally, the cellular lysates were centrifuged, and the supernatant was collected. The concentration of the proteins was determined by Bradford protein assay.

Cu(I)-Catalyzed Cycloaddition/Click Chemistry. Briefly, 20 μM rhodamine azide for in-gel labelling or biotin-azide for streptavidin enrichment was added to the samples labelled by **HTP**. This was followed by addition of 40 μM Tris(3-hydroxypropyltriazolylmethyl)amine, 0.4 mM Tris(2-carboxyethyl)phosphine and 0.4 mM $CuSO_4$. The reaction was incubated at room temperature for 2 h.

In-Gel Fluorescence Scanning. After click chemistry reaction with rhodamine-azide, chilled acetone was added to the mixture to precipitate the proteins. The proteins were then washed with cold methanol. The air-dried pellet was dissolved and heated at 95 °C for 10 min with 1 x SDS loading buffer, and resolved by SDS-PAGE. The labeled proteins were visualized by scanning the gel on a FLA-9000 Fujifilm system (excitation wavelength: 532 nm).

Streptavidin Affinity Enrichment of Biotinylated Proteins. After click chemistry reaction of **HTP** with biotin-azide, chilled acetone was added to the mixture for the proteins to precipitate. The mixture was then washed twice with cold methanol. The air-dried protein pellet was dissolved in PBS containing less than 1% SDS by vortexting and heating. Streptavidin magnetic beads (New England Biolabs) were added with gentle shaking for 4 h at room temperature to bind the biotinylated proteins. The beads were collected and washed with 5% SDS/PBS, 1% SDS/PBS and PBS to remove non-specific binding. The enriched proteins were eluted/denatured with 1 x SDS loading buffer by boiling for 20 min at 95 °C.

Western Blotting. For Western blotting experiments, samples were resolved by SDS–PAGE and transferred to a Immobilon-FL poly(vinylidene difluoride) membrane. The membrane was then blocked with 5% non-fat milk in TBST (0.1% Tween in Tris-buffered saline) for 1.5 h at room temperature. Next, the membrane was incubated with the corresponding primary antibody at 4 °C overnight. After incubation, the membrane was washed with TBST (5 × 5 min with gentle shaking) at room temperature, and then incubated with an appropriate secondary antibody (IRDye 680RD Donkey anti-Rabbit IgG) at room temperature for 1 h. Subsequently the membrane was washed with TBST (5 × 5 min with gentle shaking). Finally, the membrane was stored in TBS (Tris-buffered saline) solution and applied for scanning.

ASSOCIATED CONTENT

Supporting Information. Supporting Information can be found via the Internet at http://pubs.acs.org. The supplementary information includes experimental details, chemical synthesis and NMR spectra.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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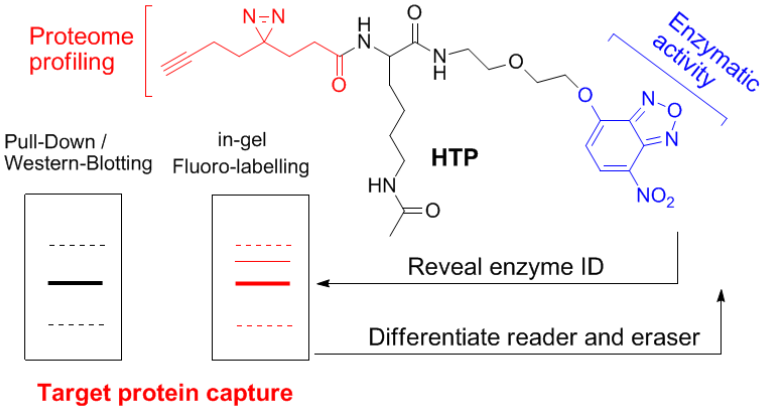
ABBREVIATIONS

HDAC, histone deacetylase; Sirt1, Sirtuin 1; TLC, thin layer chromatography.

REFERENCES

(1) (a) Zhao, Y.; Garcia, B. A. *Cold Spring Harbor perspect. Biol.* **2015**, *7*, a025064; (b) Yang, T.; Li, X. M.; Bao, X.; Fung, Y. M.; Li, X. D. *Nat. Chem. Biol.* **2016**, *12*, 70.
(2) (a) Musselman, C. A.; Lalonde, M. E.; Cote, J.; Kutateladze, T. G. *Nat. Struct. Biol.* **2012**, *19*, 1218; (b) Gillette, T. G.; Hill, J. A. *Circ. Res.* **2015**, *116*, 1245.
(3) (a) Corminboeuf, C.; Hu, P.; Tuckerman, M. E.; Zhang, Y. *J. Am. Chem. Soc.* **2006**, *128*, 4530; (b) Smith, B. C.; Hallows, W. C.; Denu, J. M. *Chem. Biol.* **2008**, *15*, 1002; (c) Gallinari, P.; Di Marco, S.; Jones, P.; Pallaoro, M.; Steinkuhler, C. *Cell Res.* **2007**, *17*, 195; (d) Leipe, D. D.; Landsman, D. *Nucl. Acids Res.* **1997**, *25*, 3693; (e) Wolffe, A. P. *Science* **1996**, *272*, 271.
(4) (a) Houtkooper, R. H.; Pirinen, E.; Auwerx, J. *Nat. Rev. Mol. Cell Biol.* **2012**, *13*, 225; (b) Gregoretti, I. V.; Lee, Y. M.; Goodson, H. V. *J. Mol. Biol.* **2004**, *338*, 17.
(5) (a) Watroba, M.; Szukiewicz, D. *Adv. Med. Sci.* **2016**, *61*, 52; (b) Holbert, M. A.; Marmorstein, R. *Curr. Opin. Struct. Biol.* **2005**, *15*, 673.
(6) (a) Madsen, A. S.; Olsen, C. A. *Angew. Chem., Int. Ed.* **2012**, *51*, 9083; (b) Bao, X.; Wang, Y.; Li, X.; Li, X.; Liu, Z.; Yang , T.; Wong, C. F.; Zhang, J.; Hao, Q.; Li, X. D. *eLife* **2014**, *3*; (c) Tan, M.; Luo, H.; Lee, S.; Jin, F.; Yang, J. S.; Montellier, E.; Buchou, T.; Cheng, Z.; Rousseaux, S.; Rajagopal, N.; Lu, Z.; Ye, Z.; Zhu, Q.; Wysocka, J.; Ye, Y.; Khochbin, S.; Ren, B.; Zhao, Y. *Cell* **2011**, *146*, 1016.
(7) (a) Xie, Z.; Dai, J.; Dai, L.; Tan, M.; Cheng, Z.; Wu, Y.; Boeke, J.; Zhao, Y. *Mol. Cell. Proteomics* **2012**, *11*, 100; (b) Du, J.; Zhou, Y.; Su, X.; Yu, J. J.; Khan, S.; Jiang, H.; Kim, J.; Woo, J.; Kim, J. H.; Choi, B. H.; He, B.; Chen, W.; Zhang, S.; Cerione, R. A.; Auwerx, J.; Hao, Q.; Lin, H. *Science* **2011**, *334*, 806.
(8) KOMATSU, T.; URANO, Y. *Anal. Sci.* **2015**, *31*, 257.
(9) Lu, X.; Wang, L.; Yu, C.; Yu, D.; Yu, G. *Front. Cell. Neurosci.* **2015**, *9*, 226.
(10) Steffan, J. S.; Bodai, L.; Pallos, J.; Poelman, M.; McCampbell, A.; Apostol, B. L.; Kazantsev, A.; Schmidt, E.; Zhu, Y.-Z.; Greenwald, M.; Kurokawa, R.; Housman, D. E.; Jackson, G. R.; Marsh, J. L.; Thompson, L. M. *Nature* **2001**, *413*, 739.
(11) Barnes, P. J. *Expert Opin. Ther. Targets* **2005**, *9*, 1111.
(12) Royce, S. G.; Ververis, K.; Karagiannis, T. C. *Ann. Clin. Lab Sci.* **2012**, *42*, 338.
(13) (a) Falkenberg, K. J.; Johnstone, R. W. *Nat. Rev. Drug Disc.* **2014**, *13*, 673; (b) Ueki, N.; Lee, S.; Sampson, N. S.; Hayman, M. J. *Nat. Commun.* **2013**, *4*, 2735.
(14) Kölle, D.; Brosch, G.; Lechner, T.; Lusser, A.; Loidl, P. *Methods* **1998**, *15*, 323.
(15) Herman, D.; Jenssen, K.; Burnett, R.; Soragni, E.; Perlman, S. L.; Gottesfeld, J. M. *Nat. Chem. Biol.* **2006**, *2*, 551.
(16) Jamonnak, N.; Hirsch, B. M.; Pang, Y.; Zheng, W. *Bioorg. Chem.* **2010**, *38*, 17.
(17) Huang, R.; Holbert, M. A.; Tarrant, M. K.; Curtet, S.; Colquhoun, D. R.; Dancy, B. M.; Dancy, B. C.; Hwang, Y.; Tang, Y.; Meeth, K.; Marmorstein, R.; Cole, R. N.; Khochbin, S.; Cole, P. A. *J. Am. Chem. Soc.* **2010**, *132*, 9986.
(18) Wu, H. W.; Liu, S. C.; Zhu, W. P.; Jiang, J. H.; Shen, G. L.; Yu, R. Q. *Electroanal* **2012**, *24*, 2365.
(19) Minoshima, M.; Kikuchi, K. *Anal. Sci.* **2015**, *31*, 287.
(20) (a) Wegener, D.; Wirsching, F.; Riester, D.; Schwienhorst, A. *Chem. Biol.* **2003**, *10*, 61; (b) Hoffmann, K.; Brosch, G.; Loidl, P.; Jung, M. *Nucl. Acids Res.* **1999**, *27*, 2057.
(21) (a) Baba, R.; Hori, Y.; Mizukami, S.; Kikuchi, K. *J. Am. Chem. Soc.* **2012**, *134*, 14310; (b) Baba, R.; Hori, Y.; Kikuchi, K. *Chem. Eur. J.* **2015**, *21*, 4695.
(22) (a) Rooker, D. R.; Buccella, D. *Chem. Sci.* **2015**, *6*, 6456; (b) Gober, I. N.; Waters, M. L. *J. Am. Chem. Soc.* **2016**, *138*, 9452.
(23) (a) Rao, V. S.; Srinivas, K.; Sujini, G. N.; Kumar, G. N. *Int. J. Proteomics* **2014**, *2014*, 147648; (b) Cravatt, B. F.; Wright, A. T.; Kozarich, J. W. *Annu. Rev. Biochem.* **2008**, *77*, 383; (c) Lee, J.-S.; Yoo, Y.-H.; Yoon, C. N. *BMB Rep.* **2014**, *47*, 149; (d) Lee, J.; Bogyo, M. *Curr. Opin. Chem. Biol.* **2013**, *17*, 118; (e) Pham, N. D.; Parker, R. B.; Kohler, J. J. *Curr. Opin. Chem. Biol.* **2013**, *17*, 90. (f) Su, Y.; Ge, J.; Zhu, B.; Zheng, Y.-G.; Zhu, Q.; Yao, S. Q. *Curr. Opin. Chem. Biol.* **2013**, *17*, 768–775.
(24) (a) Yamaguchi, T.; Asanuma, M.; Nakanishi, S.; Saito, Y.; Okazaki, M.; Dodo, K.; Sodeoka, M. *Chem. Sci.* **2014**, *5*, 1021; (b) Zhou, J.; Du, X.; Li, J.; Yamagata, N.; Xu, B. *J. Am. Chem. Soc.* **2015**, *137*, 10040; (c) Zhuang, Y. D.; Chiang, P. Y.; Wang, C. W.; Tan, K. T. *Angew. Chem., Int. Ed.* **2013**, *52*, 8124.
(25) (a) Kalesh, K. A.; Shi, H.; Ge, J.; Yao, S. Q. *Org. Biomol. Chem.* **2010**, *8*, 1749; (b) Nomura, D. K.; Dix, M. M.; Cravatt, B. F. *Nat. Rev. Cancer* **2010**, *10*, 630; (c) Li, Z.; Wang, D.; Li, L.; Pan, S.; Na, Z.; Tan, C. Y.; Yao, S. Q. *J. Am. Chem. Soc.* **2014**, *136*, 9990; (d) Li, Z.; Hao, P.; Li, L.; Tan, C. Y.; Cheng, X.; Chen, G. Y.; Sze, S. K.; Shen, H. M.; Yao, S. Q. *Angew. Chem., Int. Ed.* **2013**, *52*, 8551.
(26) Lain, S.; Hollick, J. J.; Campbell, J.; Staples, O. D.; Higgins, M.; Aoubala, M.; McCarthy, A.; Appleyard, V.; Murray, K. E.; Baker, L.; Thompson, A.; Mathers, J.; Holland, S. J.; Stark, M. J. R.; Pass, G.; Woods, J.; Lane, D. P.; Westwood, N. J. *Cancer Cell* **2008**, *13*, 454.
(27) Hallows, W. C.; Lee, S.; Denu, J. M. *Proc. Natl. Acad. Sci. U. S. A.* **2006**, *103*, 10230.
(28) Zhang, C.; Tan, C. Y. J.; Ge, J.; Na, Z.; Chen, G. Y. J.; Uttamchandani, M.; Sun, H.; Yao, S. Q. *Angew. Chem., Int. Ed.* **2013**, *52*, 14060.


Proteome profiling
Enzymatic activity
Pull-Down / Western-Blotting
in-gel Fluoro-labelling
HTP
Reveal enzyme ID
Differentiate reader and eraser
Target protein capture