A chemical catalyst enabling histone acylation with endogenous Acyl-CoA

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Abstract

Post-translational modifications (PTMs) of proteins, e.g., epigenetic acetylation of lysine residues in histones, are crucial to cellular functions and related to diseases. Chemical tools to directly introducing epigenetic lysine acetylation hold promise for elucidating the PTM's functions and treating diseases. Although several chemical catalysts introducing protein acetylation in live cells were reported, there is no precedent promoting in-cell acetylation of epigenetically important but often low-reactive histone proteins using endogenous acetyl-CoA, as histone acetyltransferases (HATs) do. Herein, we developed a chemical catalyst *m*BnA enabling selective in-cell histone lysine acylation (H2BK120ac) using endogenous acyl-CoA as a sole acyl donor. A hydroxamic acid of proper electronic characteristics as a nucleophilic catalytic site combined with a thiol-thioester exchange process enabled *m*BnA to activate low concentration of acyl-CoAs in cells, promoting histone lysine acylations (acetylation and malonylation). This chemical catalyst will be a small-molecule surrogate to HAT and thus a unique tool to synthetic epigenetics.

Introduction

Post-translational modifications (PTMs) of proteins play important roles in eukaryotic cellular processes to determine proteins' activity state, localization, turnover, and interactions with other proteins[1]. Among PTMs, acetylation of lysine residues neutralizes the positively charged ε-amino group and produces profound effects on protein structures, functions, or stability. Acetylation of histone lysine residues is one of the fundamental epigenetic modifications and plays a pivotal role in regulating gene transcription. Histone proteins are lysine-rich and wrapped by DNA to densely pack genetic information primarily through electrostatic interactions into cellular chromatin[2]. It is known that disorders of the tightly regulated histone lysine acetylation levels are related to diseases[3,4].

Lysine acetylation level of protein in cells, including histone, is tightly regulated by two-types of enzymes, lysine acetyltransferases (KATs) and lysine deacetylases (KDACs)[5,6]. KATs promote acetyl-transfer reactions from acetyl-CoA (Ac-CoA) to lysine residues, while non-enzymatic acetylation also proceeds in some cases, such as in the mitochondrial environment (high Ac-CoA concentrations and high pH) or for particularly reactive lysine residues[7–9]. Although small molecule inhibitors of KDACs to indirectly promote protein acetylation levels are approved as drugs (e.g., vorinostat and romidepsin)[10,11], a way of directly promoting histone acetylation level is rare[12]. Chemical methods that can promote histone lysine acetylation in place of enzymes should prove useful as a tool for understanding and treating the diseases.

We previously reported a mercaptomethyl group-tethered 4-dimethylaminopyridine (DMAP) catalyst, DSH, for histone lysine acetylation (**Fig. 1a**)[13]. With thioester acetyl donors including Ac-CoA,

nucleosome ligand-conjugated DSH promoted regioselective histone acetylation reaction in recombinant nucleosomes (with Ac-CoA)[13] and in live cells (with a cell permeable truncated version of Ac-CoA: NAC-Ac)[14,15] through a thiol-thioester exchange between DSH and the thioester donors followed by a intramolecular nucleophilic activation of the acetyl group. This resulted in the first live-cell epigenome manipulation with a chemical catalyst[15]. However, DSH requires addition of over ten millimolar concentrations of exogenous thioester acetyl donors for the high-yielding in-cell reaction. When applying the DSH catalyst without the exogenous acetyl donor to in-cell acetylation of proteins, no detectable promotion of protein acetylation was observed, suggesting that the catalyst activity of DSH was not sufficient to activate endogenous Ac-CoA present at low concentration in living cells.

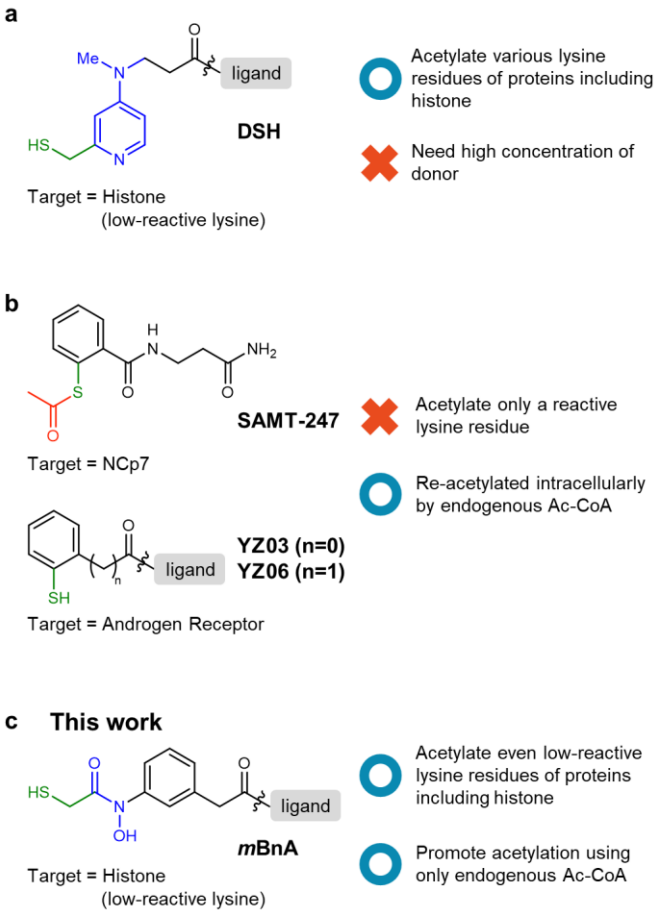

Fig. 1 Chemical catalysts to acetylate protein lysine residues using Ac-CoA. a DSH catalyst, which can acetylate low-reactive histone lysine residues. **b** Thiosalicylamide-type catalyst and its derivatives. They can acetylate only reactive lysine residues with endogenous Ac-CoA in living cells. **c** *m*BnA catalyst in this work, which can activate endogenous Ac-CoA to acetylate low-reactive lysine residues, such as that in histone, in living cells.

Chemical compounds that promote lysine acetylation of non-histone proteins only with endogenous Ac-CoA in living cells were reported. SAMT-247 (**Fig. 1b**) is an inhibitor of nucleocapsid protein NCp7, which is one of the attractive targets for antiretroviral HIV therapy. Acetyl group of SAMT-247 is first

transferred to a cysteine residue (Cys36) of NCp7, and the acetylated cysteine further transfers its acetyl group to a lysine residue at the zinc finger of the protein, which eventually resulted in ejection of the essential zinc ion. The des-acetylated thiol group of SAMT-247 was reported to be re-acetylated by endogenous Ac-CoA[16–18]. Based on SAMT-247, Zhang et al. developed thiosalicylamide-tolfenamic acid conjugate YZ03 and its derivative YZ06 (**Fig. 1b**) as ligand-directed acetylation catalysts for androgen receptor (AR)[19]. YZ03 selectively acetylated K720 of AR, which is located at the ligand binding domain of AR, with endogenous Ac-CoA, resulting in inhibition of the coactivator peptide association. The authors stated that K720 is a specially reactive lysine due to the decreased pK_a caused by nearby basic residues, which is the reason for the selective acetylation over other lysine residues more proximal to the ligand binding site. Since lysine residues undergoing PTM regulations, such as those of histones, are often less reactive[20], a chemical catalyst capable of promoting lysine acetylation independently on the local amino acid environment (e.g., Cys to Lys acetyl transfer in the case of NCp7) or intrinsic high reactivity (e.g., K720 of AR) is necessary to intervene in cellular PTM systems, such as epigenetic lysine acetylation of histones.

Here, we report the first chemical catalyst promoting lysine acetylation of the epigenetically important histone protein in living cells using endogenous Ac-CoA as a sole acetyl donor. The use of hydroxamic acid as a nucleophilic catalyst core, instead of 4-dimethylaminopyridine of DSH, and structural optimization based on kinetic experiments afforded the optimal *m*BnA catalyst, which enabled activation of endogenous acyl-CoAs and in-cell lysine acylation of low-reactive proteins, such as histone (**Fig. 1c**).

Results

<**Profiling reactivity difference of lysine residues**>

The example of thiosalicylamide-type catalysts (SAMT-247 and YZ03/06) indicates that the acetylation behavior of catalyst is strongly dependent on the reactivity of a target lysine residue. To compare the catalytic activity toward lysine residues with different reactivity, we first examined the reactivity of the lysine residues of two proteins: K32 of *E. coli* dihydrofolate reductase (eDHFR), which is often used as a substrate in ligand-directed catalysis[14,21], and histone H2BK120, which is a target of our epigenetic manipulation by chemical catalysis[15]. We used amine-reactive sulfotetrafluorophenyl (STP) pentynoate as a probe for quantitative lysine reactivity analysis[20]. Recombinant proteins were incubated with STP pentynoate and acylation yield of each lysine residue was determined by liquid chromatography-tandem mass spectrometry (LC−MS/MS) analysis (**Fig. 2a**, **Supplementary Fig. 1a**). While eDHFR K32 was acylated in 42% yield, the acylation yield of histone H2BK120 was only 5%. This revealed that H2BK120 has much lower reactivity to acylation than eDHFR K32, and provided us an assay platform for investigating the difference of the catalysts' performance between high-reactive and low-reactive lysine residues in the following studies.

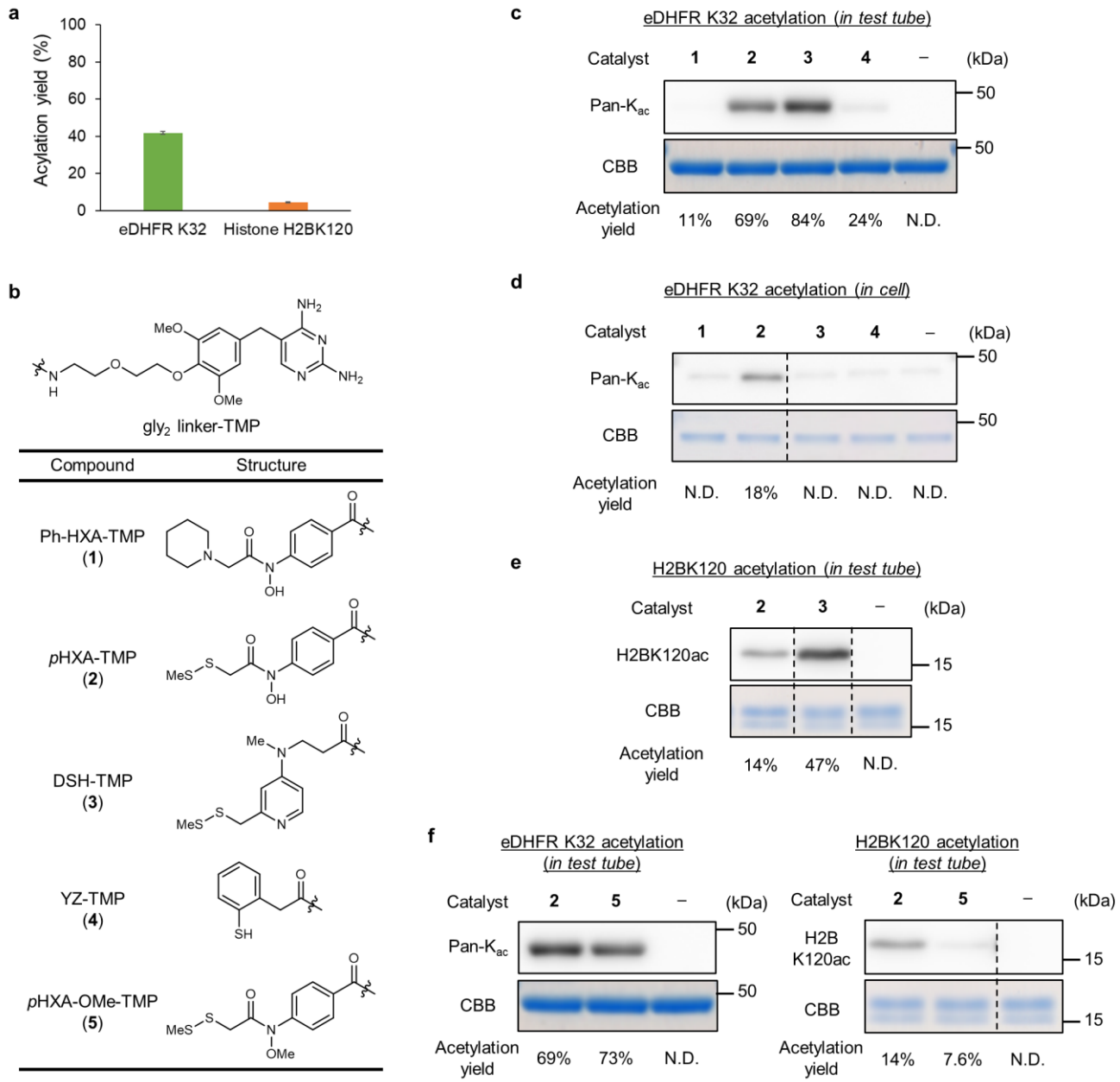

Fig. 2 The 1st generation catalyst *p*HXA can activate Ac-CoA but only acetylate relatively high-reactive lysine residue. a Comparison of the reactivity of lysine residues. The reactivity of eDHFR K32 and Histone H2BK120 was quantified based on the acylation yield using the STP pentynoate probe. Error bars denote range (n=2). **b** Chemical structures of the catalysts. **c** Acetylation of eDHFR K32 in a test tube. Recombinant eDHFR-GFP (5 μM) was reacted with each catalyst (50 μM) in the presence of Ac-CoA (1 mM) and TCEP (200 μM) at 37 °C for 2 h, and its acetylation was detected with pan-K_{ac}

antibody by western blot analysis. Total eDHFR-GFP protein was visualized with Coomassie Brilliant Blue (CBB) staining. The acetylation yields at eDHFR K32 were quantified with LC-MS/MS analysis. **d** Acetylation of eDHFR K32 in living cells. eDHFR-GFP was expressed in HEK293T cells and the cells were treated with the catalysts (100 μM) for 10 h. eDHFR-GFP was immunoprecipitated from cell lysate, and acetylation was detected with pan-K_{ac} antibody by western blot analysis. Total eDHFR-GFP protein was visualized with CBB staining. The acetylation yields at eDHFR K32 were quantified with LC-MS/MS analysis. **e** Acetylation of histone H2BK120 in a test tube. Recombinant mono-nucleosome (0.35 μM) was reacted with each catalyst (5 μM) in the presence of Ac-CoA (1 mM), LieD protein ligand (2 μM), and TCEP (200 μM) at 37 °C for 5 h. Histone acetylation was detected with anti-H2BK120ac antibody by western blot analysis. Total histone proteins were visualized with CBB staining. The acetylation yields at H2BK120 were quantified with LC-MS/MS analysis. **f** Catalytic activity of negative control catalyst *p*HXA-OMe-TMP (**5**) toward eDHFR K32 (left) and histone H2BK120 (right) in test tube reactions with Ac-CoA. Acetylation of each lysine residue was detected with pan-K_{ac} or anti-H2BK120ac antibodies, and total proteins were visualized by CBB staining. The acetylation yields at eDHFR K32 or H2BK120 were quantified with LC-MS/MS analysis.

<**The 1st generation catalyst *p*HXA that can activate endogenous Ac-CoA**>

To develop an active catalyst that can acetylate low reactive lysine residues, such as histone H2BK120, with endogenous Ac-CoA, we started a catalyst development campaign based on the thiol-thioester

exchange mechanism. We previously reported that DSH undergoes thiol-thioester exchange with a thioester acetyl donor faster than the following process involving intramolecular nucleophilic activation of the thioacetyl group on the catalyst and subsequent acetyl transfer to a target lysine residue (**Supplementary Fig. 1b**)[22]. Considering that concentration of the *S*-acetylated catalyst is lower with low concentration of Ac-CoA, a more active nucleophilic catalyst center is required. DMAP, the nucleophilic core of DSH, is a basic catalyst, and thus is mostly deactivated by protonation under physiological conditions (i.e., neutral aqueous conditions)[23]. We previously reported that a piperidine-conjugated hydroxamic acid Ph-HXA **1** (**Fig. 2b**) is deprotonated under physiological conditions and serves as a superior nucleophilic catalyst to DMAP[24]. Based on these considerations, we designed *p*HXA-catalyst **2** (**Fig. 2b**), which contains a hydroxamic acid moiety and a masked thiol group as a nucleophilic center and a handle to capture acetyl group from Ac-CoA, respectively (*note*: the disulfide is cleaved under reducing conditions[14], **Supplementary Fig. 1c**–**d**).

The catalyst *p*HXA was applied to the acetylation reaction of eDHFR K32. *p*HXA was conjugated to trimethoprim (TMP), a ligand to eDHFR, to deliver the catalyst to K32 of eDHFR (**Fig. 2b**)[14]. The catalyst was mixed with purified eDHFR-GFP protein and Ac-CoA in the presence of tris(2-carboxyethyl)phosphine hydrochloride (TCEP) in a test tube. Promotion of eDHFR acetylation was analyzed with western blot using a pan anti-acetyllysine (K_{ac}) antibody and quantified with LC-MS/MS analysis (**Fig. 2c**). The *p*HXA catalyst **2** promoted acetylation of eDHFR in 69% yield, and its modified site was identified at K32 (**Supplementary Fig. 2a**). The TMP-conjugates of the other previously reported

catalysts, DSH-TMP **3** and YZ-TMP **4**, also promoted the acetylation of eDHFR (84% and 24% respectively), while Ph-HXA **1**, which lacks a thiol group, acetylated eDHFR with Ac-CoA only in low yield (11%).

Then we tested eDHFR acetylation reaction within living cells. HEK293T cells transfected with eDHFR-GFP were treated with TMP-conjugated catalysts without addition of any exogenous donors. After the reaction, the cells were lysed and eDHFR-GFP was purified with anti-GFP magnetic beads. Western blot and LC-MS/MS analyses revealed that *p*HXA **2** was the only catalyst to promote acetylation of eDHFR protein (in 18% yield at K32, **Fig. 2d**). No promotion of acetylation was observed with other Ac-CoA-activating catalysts possibly due to inefficient activation of the low concentration of endogenous acetyl donor (for DSH) and/or insufficient catalyst activity (for YZ). These results suggest that *p*HXA is an active catalyst to acetylate a protein lysine residue in living cells without the need for exogenous donors.

<***p*HXA cannot efficiently acetylate low reactive lysine residues**>

Next, we tested the acetylation activity of *p*HXA for a low reactive lysine residue, histone H2BK120. For selective targeting of catalyst-TMP conjugates to H2BK120, LANA (latency-associated nuclear antigen of Kaposi's sarcoma-associated herpesvirus) peptide-inserted eDHFR (LieD) system we previously reported was used[25]. In this system, the LieD protein complexed with a catalyst-TMP conjugate delivers the catalyst to the acidic patch of the nucleosome, to promote acetylation of the proximal H2BK120 (**Supplementary Fig. 2b**). The catalyst-TMP conjugates, purified LieD protein, and Ac-CoA were

incubated with recombinant nucleosome in a test tube, and then acetylation of H2BK120 was analyzed with western blot using an anti-H2BK120ac antibody. As a result, *p*HXA-TMP **2** promoted acetylation of H2BK120, but its acetylation activity was unexpectedly lower than that of DSH-TMP **3** (14% for *p*HXA **2** vs 47% for DSH **3**, **Fig. 2e**). To further investigate the reactivity details of *p*HXA, we synthesized *O*-methylated derivative *p*HXA-OMe **5**, which cannot generate the putative active *O*-acetyl species, and compared its acetylation activity with that of *p*HXA **2** (**Fig. 2f**). In acetylation reaction of the reactive eDHFR K32, *p*HXA-OMe **5** showed nearly the same level of acetylation activity as *p*HXA **2**. In contrast, *p*HXA-OMe **5** hardly promoted acetylation of the low reactive H2BK120. These results suggested two points: 1) Generation of only the *S*-acetylated catalyst is sufficient to react with the reactive eDHFR K32, 2) *p*HXA barely generates the active *O*-acetylated intermediate, which would be necessary to acetylate low reactive lysine residues, such as H2BK120, and thus failed H2BK120 acetylation. This indicated that *p*HXA is not a suitable catalyst to acetylate low-reactive lysine residues and prompted us to improve its catalytic activity through structural optimization.

<**Structural optimization of *p*HXA to identify an active acetylation catalyst *m*BnA for lysine residues**>

Based on the assumption above, we first quantified the formation of the *S*- and *O*-acetylated intermediates from the *p*HXA catalyst with high-performance liquid chromatography (HPLC) and liquid chromatography-mass spectrometry (LC–MS). The *p*HXA-TMP **2** (25 μM) and Ac-CoA (5 mM) were

mixed in tris buffer (pH 7.5) in the presence of TCEP (200 μM), and the formation of the *S*- and *O*-acetylated catalysts was monitored. As a result, while the *S*-acetylated intermediate was smoothly and sufficiently formed to reach the equilibrium within 2 h (87% in 3 h), only a little amount of the *O*-acetylated intermediate (2.0% in 3 h) was formed, which was consistent with our assumption (**Fig. 3a**–**b, Supplementary Fig. 3**). We supposed that the *O*-acetylated intermediate is too unstable to form due to high acidity of the hydroxamic acid moiety in *p*HXA.

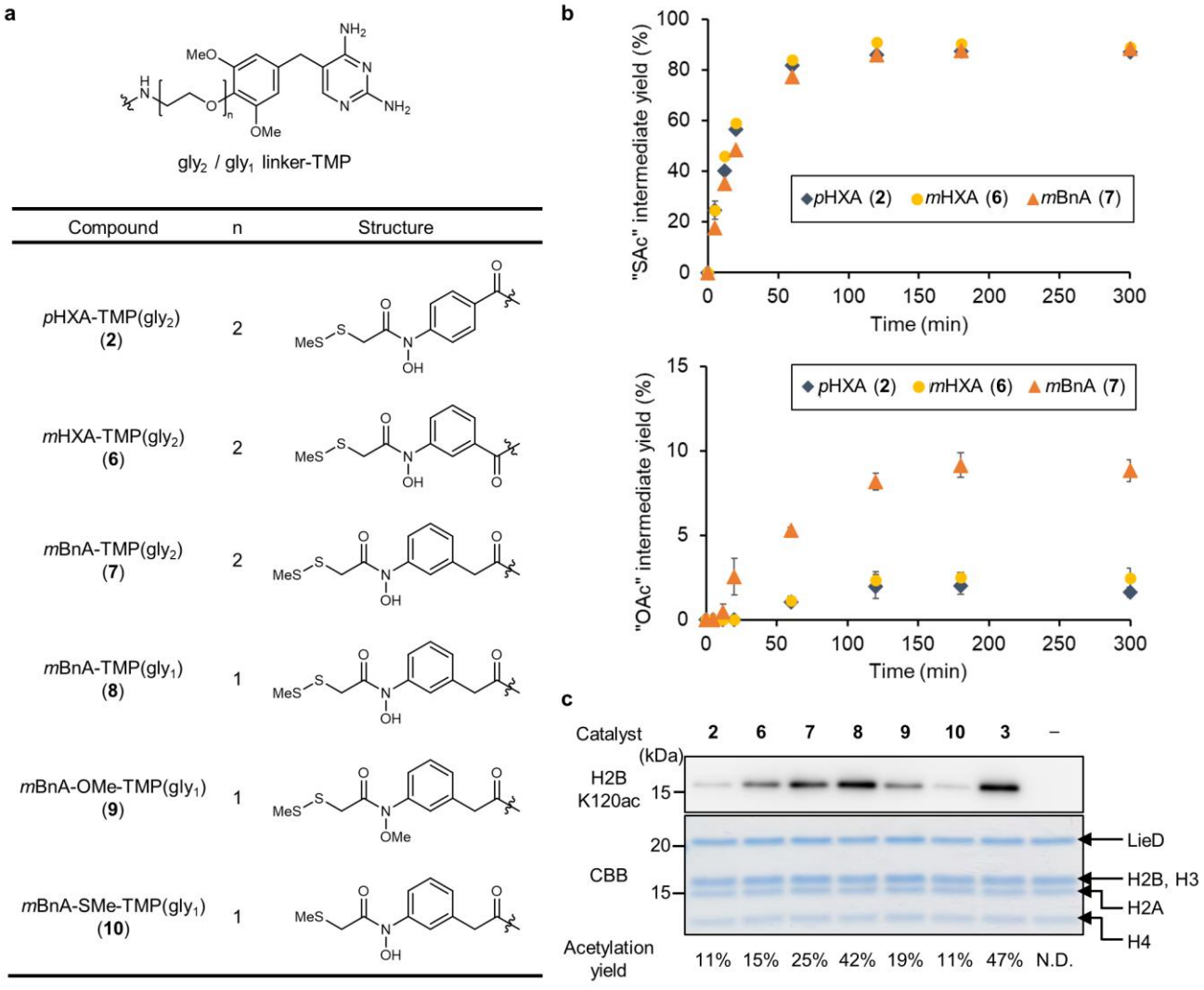

Fig. 3 Structural optimization of *p*HXA to identify an active acetylation catalyst *m*BnA for low-reactive lysine residues. a Chemical structures of the catalysts. **b** Generation of *S*- (upper) and *O*-acetylated (lower) intermediates of the indicated catalysts. Each catalyst (25 μM) was reacted with Ac-CoA (5 mM) in the presence of TCEP (200 μM) at 37 °C for the indicated time, and the acetylated intermediates were quantified by HPLC analysis. Error bars denote range (n=2). **c** Acetylation of histone H2BK120 in test tube reactions. Recombinant mono-nucleosome (0.35 μM) was reacted with each catalyst (5 μM) in the presence of Ac-CoA (1 mM), LieD protein ligand (2 μM) and TCEP (200 μM) at 37 °C for 5 h, and its acetylation was detected with anti-H2BK120ac antibody by western blot analysis. Total histones and LieD proteins were visualized with CBB staining. The acetylation yields at H2BK120 were quantified with LC-MS/MS analysis.

We, therefore, designed *m*HXA **6** (**Fig. 3a**) which has a carbonyl group at the 3-position of the aromatic ring, and *m*BnA **7**, which has an additional methylene group inserted between the aromatic ring and the amide carbonyl group, to reduce the acidity of the hydroxamic acid. Both catalysts underwent ready thiol-thioester exchange and reached the equilibrium within 2 h, as was observed for *p*HXA **2**. While *m*HXA-TMP **6** afforded only slightly improved *O*-acetylated intermediate formation (2.5% for *m*HXA **6** vs 2.0% for *p*HXA **2** in 3 h), *m*BnA-TMP **7** showed its substantially improved formation (9.1% in 3 h). Measurement of pK_a values of hydroxamic acid moieties in *p*HXA and *m*BnA with ultraviolet visible absorption (UV-Vis) spectroscopy revealed that the pK_a of *p*HXA and *m*BnA are 8.3 and 8.6 respectively,

which supports our notion that the acidity of the hydroxamic acid is the key to facile generation of the *O*-acetylated catalyst (**Supplementary Fig. 4a**–**b**)[24].

Catalytic activities of the new catalysts were tested in histone H2BK120 acetylation reactions with the LieD system in test tubes (**Fig. 3c**). Both *m*HXA-TMP **6** and *m*BnA-TMP **7** promoted H2BK120 acetylation more efficiently than *p*HXA-TMP **2**, and the reactivity order was consistent with the ability to generate the *O*-acetylated intermediates, which suggested that the generation of the *O*-acetylated intermediate is important to acetylate the low reactive H2BK120. Truncation of the linker between TMP and the *m*BnA catalyst further improved the H2BK120 acetylation ability to afford the optimized catalyst *m*BnA-TMP(gly_1) **8** (42% yield, **Fig. 3a**, **c**). Negative control catalysts, *O*-methylated and *S*-methylated derivatives of *m*BnA-TMP (**9**, **10**) did not promote the H2BK120 acetylation as efficiently as the optimized catalyst **8**, which demonstrated that both the hydroxamic acid moiety and the thiol group were essential for the acetylation ability of the *m*BnA catalyst, as expected from the postulated reaction mechanism (**Supplementary Fig. 1d**).

<**In-cell histone H2BK120 acetylation without exogenous acetyl donor**>

With the optimized catalyst *m*BnA-TMP(gly_1) **8** in hand, we tried in-cell acetylation reaction of histone H2BK120 without exogenous donors. LieD-transfected HEK293T cells were treated with catalysts alone (100 μM) for 10 h, and then histone acetylation levels were analyzed by western blot (**Fig. 4a**). Previously reported catalysts, Ph-HXA-TMP **1**[24], DSH-TMP **3**[14] and YZ-TMP **4**[19] failed to promote acetylation of

H2BK120 in living cells without exogenous acetyl donors (lane 1, 7 and 8). The optimized catalyst *m*BnA-TMP **8**, however, promoted H2BK120 acetylation (2.5% yield, lane 3). The acetylation efficiency of *p*HXA-TMP **2** and *O*- and *S*-methylated *m*BnA-TMP catalysts (**9**, **10**) were much lower than that of **8**, which is consistent with the results in test tubes (**Fig. 3d**). The promotion of the H2BK120 acetylation by *m*BnA-TMP **8** was suppressed by the addition of TMP as a binding competitor (lane 6), indicating that the acetylation was due to proximity effects of the catalyst. Acetylation level at H3K9 and H3K18, which are the substrates of KAT[26], did not change by the treatment with *m*BnA-TMP **8** (**Supplementary Fig. 5**). These two results indicate that the promotion of the H2BK120 acetylation by *m*BnA catalyst was not dependent on cellular enzymatic activities, but was caused by the chemical catalyst in cells.

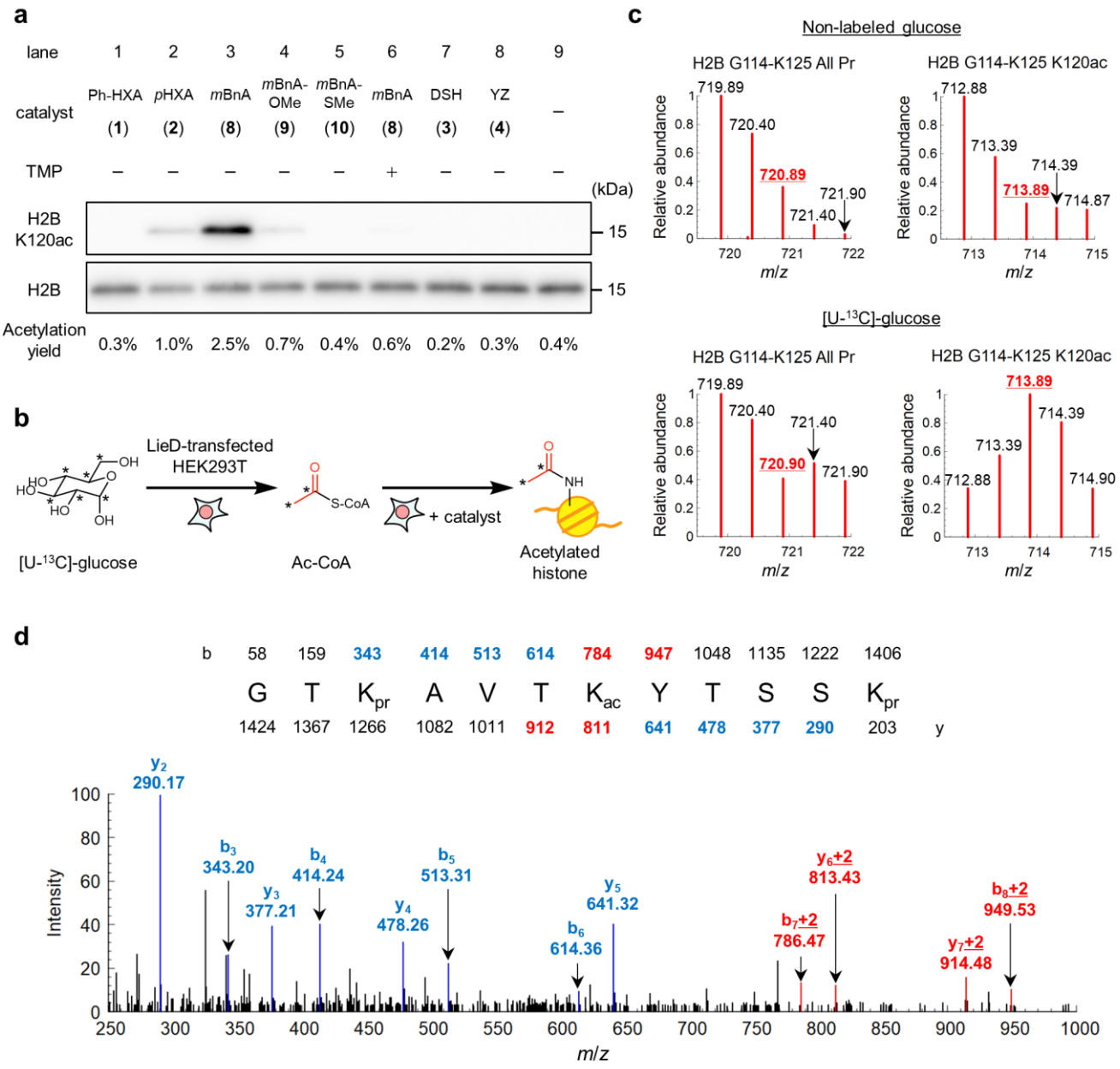

Fig. 4 The optimized catalyst *m*BnA can acetylate a histone lysine residue in living cells using endogenous Ac-CoA. a Acetylation of histone H2BK120 in living cells. LieD-transfected HEK293T cells were treated with the indicated catalysts (100 μM) for 10 h. Histone proteins were acid-extracted, and H2BK120 acetylation and total H2B subunit were detected with anti-H2BK120ac and anti-H2B antibodies respectively by western blot analysis. The acetylation yields at H2BK120 were quantified with LC-MS/MS analysis. **b** Schematic illustration of metabolic isotopic labeling of Ac-CoA using [U-^{13}C]-glucose. The *U*

refers to universally labeled (i.e., all carbons are ^{13}C). An asterisk denotes the position of ^{13}C-incorporation. **c** Detection of isotopic labeling at acetylated H2BK120. LieD-transfected HEK293T cells were incubated in medium containing non-labeled glucose (upper) or [U-^{13}C]-glucose (lower), and was treated with *m*BnA-TMP(gly_1) **8**. Acid-extracted histones were treated with propionic anhydride to propionylate non-acetylated lysine residues, digested by trypsin and Glu-C peptidases, and then analyzed by LC–MS/MS. The $[M + 2H]^{2+}$ parent peaks were analyzed. Isotopic distribution of the H2B G114-K125 peptide, whose lysine residues were all propionylated (i.e., non-acetylated, All Pr) are shown in left, while that of the mono-acetylated peptide (K120ac) is shown in right. Since we observed $[M + 2H]^{2+}$ peaks, +2 Da shift was detected as +1 *m*/*z* shift as shown in red (720.90 and 713.89). **d** LC-MS/MS trace of the H2B G114-K125 K120ac peptide from LieD-transfected HEK293T cells cultured in [U-^{13}C]-glucose and treated with *m*BnA-TMP(gly_1) **8**. The sequence and the calculated *m*/*z* values of the fragment ions are shown. Peaks shown in red indicated incorporation of +2 Da shift at H2BK120.

<**Evidence of using endogenous Ac-CoA by *m*BnA**>

To confirm that *m*BnA used Ac-CoA as the endogenous donor in the in-cell H2BK120 acetylation reaction, we carried out metabolic isotopic labeling of Ac-CoA with ^{13}C-labeled glucose[27]. To cell medium without containing glucose, [U-^{13}C]-glucose was supplemented, which is metabolically converted into Ac-CoA with two isotopic labeled carbons in the acetyl group (**Supplementary Fig. 6**). First, LieD-transfected HEK293T cells were treated with [U-^{13}C]-glucose (4.5 g/L) for 16 h. The cells were, then, treated with

*m*BnA-TMP **8** for 10 h, histones were purified and digested with trypsin and Glu-C peptidases, and an expected two-Dalton mass shift at H2BK120ac was detected by LC-MS/MS analysis (**Fig. 4b–c**). In the mass traces shown in **Fig. 4c**, All Pr denotes a non-acetylated histone peptide containing H2BK120 (*note*: the unreacted free lysines are propionylated in the LC–MS/MS analysis: See Supplementary information), while K120ac denotes a peptide acetylated at H2BK120. Control samples with no glucose-labeling provide the baseline isotopic distribution for the histone peptide, and All Pr and K120ac showed the same isotopic distribution (**Fig. 4c, upper panel**). When [U-^{13}C]-glucose was used, the All Pr peptide showed a slight heavy shift of the isotopic distribution because some of the proteinogenic amino acids are biosynthesized from the [U-^{13}C]-glucose and ^{13}C were incorporated into the peptide backbone (**Fig. 4c, lower panel**). Under the conditions, the K120ac peptide showed a significant two-Dalton mass shift of the isotopic distribution. Additionally, tandem mass spectrometry analysis showed that the two-Dalton mass shifts occurred specifically in the MS/MS fragment containing the acetyl group at K120 (**Fig. 4d**). These results indicate that the endogenous acetyl donor which *m*BnA used in the H2BK120 acetylation was Ac-CoA.

<**Activation of endogenous non-acetyl acyl-CoA to acylate histone**>

Finally, we examined whether *m*BnA can activate acyl-CoA other than Ac-CoA in living cells. Cells contain various non-acetyl acyl-CoAs and their concentrations are usually lower than that of Ac-CoA[28,29]. We focused on negatively charged malonyl-CoA (Ma-CoA) as an example, and tested whether

*m*BnA-TMP **8** can activate even non-acetyl acyl-CoA to acylate histone. LieD-transfected HEK293T cells were treated with *m*BnA-TMP **8** for 10 h, and purified histones were analyzed with western blot using an anti-malonyl lysine antibody. As a result, the malonylation level of histone H2B increased in the cells treated with *m*BnA-TMP **8** (lane 1 vs 3, **Fig. 5a–b**). The malonylation level of H4, which is the major endogenous malonylation target, was not affected by the *m*BnA-TMP **8** treatment, suggesting that H2BK120 malonylation was introduced not by enzymes but by the chemical catalyst. Intracellular concentrations of acyl-CoAs are known to increase by treatment with corresponding sodium carboxylates (**Fig. 5a**)[30,31]. The cells treated with both disodium malonate (20 mM) and *m*BnA-TMP **8** showed more significant promotion of the H2BK120 malonylation, demonstrating that the *m*BnA catalyst has the ability of activating non-acetyl type endogenous acyl-CoA to acylate histone lysine residues.

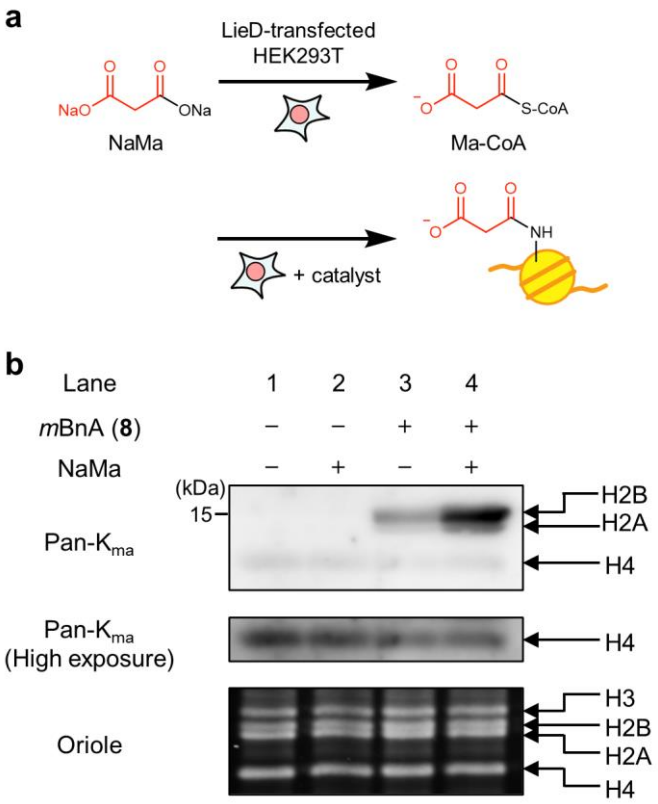

Fig. 5 The catalyst *m*BnA can activate endogenous non-acetyl acyl-CoA to acylate histone. a Schematic illustration of histone malonylation. The concentration of Ma-CoA in HEK293T cells was metabolically increased by treatment with disodium malonate (NaMa). The malonyl group of Ma-CoA was then transferred to histone H2BK120 by *m*BnA catalyst. **b** Malonylation of histone in living cells. LieD-transfected HEK293T cells was treated with *m*BnA-TMP(gly_1) **8** (100 μM) in the presence or absence of NaMa (20 mM), and histone proteins were acid-extracted. Malonylated histones were detected using pan-K_{ma} antibody by western blot analysis. Total histone proteins were visualized with oriole staining.

Discussion

We developed a novel lysine acylation catalyst *m*BnA, which can acylate a low-reactive histone protein

solely with endogenous acyl sources, acyl-CoAs. A thiol group as an acyl-CoA-capturing motif, the hydroxamic acid as a nucleophilic catalyst core, which is deprotonated and activated under physiological conditions[24,32], and structural optimization to adjust acidity of the hydroxamic acid for modulating the stability of the postulated active acyl species, acyl hydroxamate, were the keys for the success. The histone acetylation activity of *m*BnA-TMP **8** reached the same level as that of our previously developed catalyst DSH in test-tubes (**Fig. 3d**), but intriguingly its activity for the in-cell reaction with endogenous acetyl donor was clearly higher than that of DSH (**Fig. 4a**). The concentration of Ac-CoA in cells are reported to be around tens of micromolar[33,34], which is lower than that in the test-tube reactions (millimolar concentrations), and thus the concentration of the *S*-acetylated catalyst formed through a thiol-thioester exchange is expected to be low in cells. Under such circumstances, more efficient intramolecular activation of the *S*-acetyl group by the nucleophilic core should be important. Hence, *m*BnA with the superior nucleophilic center would exhibit higher acetylation activity than DSH in the in-cell reactions with endogenous acetyl donor.

Chemical histone acetylation only with endogenous Ac-CoA (i.e., without addition of exogenous acetyl donors) is favorable for synthetic manipulation of cellular epigenome. The lack of need for exogenous acetyl donor would minimize undesirable perturbations to cells, considering examples where some types of thioesters (and thiols formed after hydrolysis) work as HDAC inhibitors[35,36]. Currently, *m*BnA catalyst was only applied to H2BK120 acylation. Although H2BK120 acetylation with a chemical catalyst was demonstrated to have a potential to manipulate cellular epigenome[15], catalysts capable of targeting other

histone lysine residues, such as those in histone tail domains, are also appealing. Replacing the TMP motif of *m*BnA-TMP **8** to other nucleosome targeting motifs, such as octaarginine and pyrrole-imidazole-polyamide[13,37,38] is a way of developing such a catalyst.

Our *m*BnA catalyst was able to activate non-acetyl acyl-CoAs, such as Ma-CoA to transfer its malonyl group to a histone lysine residue. It was recently reported that carboxylic acids taken from foods and drugs are metabolized to form non-canonical acyl-CoAs in body, and the acyl groups are enzymatically or non-enzymatically incorporated into histone lysine residues to induce transcriptional perturbations[39,40]. The ability of *m*BnA to acylate histone in an acyl-CoA concentration-dependent manner could be potentially valuable in studying the effect of the exogenous carboxylic acid and the resulting non-canonical protein lysine acylations in cells.

In summary, we have developed a lysine acylation catalyst *m*BnA, which can activate a low concentration of acyl-CoA in cells to transfer its acyl group to a lysine residue of epigenetically important histone protein. This could be a first step to develop a chemical catalyst to serve as a small molecule surrogate of histone acetyl transferases for synthetic epigenome manipulations.

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References

1. Mann, M. & Jensen, O. N. Proteomic analysis of post-translational modifications. *Nat. Biotechnol.* **21**, 255–261 (2003).

2. Luger, K., Mäder, A. W., Richmond, R. K., Sargent, D. F. & Richmond, T. J. Crystal structure of the nucleosome core particle at 2.8 Å resolution. *Nature* **389**, 251–260 (1997).

3. Mirabella, A. C., Foster, B. M. & Bartke, T. Chromatin deregulation in disease. *Chromosoma* **125**, 75–93 (2016).

4. Dawson, M. A. & Kouzarides, T. Cancer Epigenetics: From Mechanism to Therapy. *Cell* **150**, 12–27 (2012).

5. Narita, T., Weinert, B. T. & Choudhary, C. Functions and mechanisms of non-histone protein acetylation. *Nat. Rev. Mol. Cell Biol.* **20**, 156–174 (2019).

6. Peserico, A. & Simone, C. Physical and Functional HAT/HDAC Interplay Regulates Protein Acetylation Balance. *J. Biomed. Biotechnol.* **2011**, 1–10 (2011).

7. Wagner, G. R. & Payne, R. M. Widespread and Enzyme-independent N^{ε}-Acetylation and N^{ε}-Succinylation of Proteins in the Chemical Conditions of the Mitochondrial Matrix. *J. Biol. Chem.* **288**,

29036–29045 (2013).

8. James, A. M. *et al.* Non-enzymatic *N*-acetylation of Lysine Residues by AcetylCoA Often Occurs via a Proximal *S*-acetylated Thiol Intermediate Sensitive to Glyoxalase II. *Cell Rep.* **18**, 2105–2112 (2017).

9. James, A. M. *et al.* The Causes and Consequences of Nonenzymatic Protein Acylation. *Trends Biochem. Sci.* **43**, 921–932 (2018).

10. Olsen, E. A. *et al.* Phase IIB multicenter trial of vorinostat in patients with persistent, progressive, or treatment refractory cutaneous t-cell lymphoma. *J. Clin. Oncol.* **25**, 3109–3115 (2007).

11. Whittaker, S. J. *et al.* Final results from a multicenter, international, pivotal study of romidepsin in refractory cutaneous T-cell lymphoma. *J. Clin. Oncol.* **28**, 4485–4491 (2010).

12. Hilton, I. B. *et al.* Epigenome editing by a CRISPR-Cas9-based acetyltransferase activates genes from promoters and enhancers. *Nat. Biotechnol.* **33**, 510–517 (2015).

13. Amamoto, Y. *et al.* Synthetic Posttranslational Modifications: Chemical Catalyst-Driven Regioselective Histone Acylation of Native Chromatin. *J. Am. Chem. Soc.* **139**, 7568–7576 (2017).

14. Hamajima, W. *et al.* Site-Selective Synthetic Acylation of a Target Protein in Living Cells Promoted by a Chemical Catalyst/Donor System. *ACS Chem. Biol.* **14**, 1102–1109 (2019).

15. Fujiwara, Y. *et al.* Live-cell epigenome manipulation by synthetic histone acetylation catalyst system. *Proc. Natl. Acad. Sci. U.S.A.* **118**, e2019554118 (2021).

16. Jenkins, L. M. M. *et al.* Small-molecule inactivation of HIV-1 NCp7 by repetitive intracellular acyl transfer. *Nat. Chem. Biol.* **6**, 887–889 (2010).

17. Miller Jenkins, L. M. *et al.* Studies on the Mechanism of Inactivation of the HIV-1 Nucleocapsid Protein NCp7 with 2-Mercaptobenzamide Thioesters. *J. Med. Chem.* **48**, 2847–2858 (2005).

18. Miller Jenkins, L. M. *et al.* Specificity of acyl transfer from 2-mercaptobenzamide thioesters to the HIV-1 nucleocapsid protein. *J. Am. Chem. Soc.* **129**, 11067–11078 (2007).

19. Zhang, Y., Mantravadi, P. K., Jobbagy, S., Bao, W. & Koh, J. T. Antagonizing the Androgen Receptor with a Biomimetic Acyltransferase. *ACS Chem. Biol.* **11**, 2797–2802 (2016).

20. Hacker, S. M. *et al.* Global profiling of lysine reactivity and ligandability in the human proteome. *Nat. Chem.* **9**, 1181–1190 (2017).

21. Takaoka, Y., Nishikawa, Y., Hashimoto, Y., Sasaki, K. & Hamachi, I. Ligand-directed dibromophenyl benzoate chemistry for rapid and selective acylation of intracellular natural proteins. *Chem. Sci.* **6**, 3217–3224 (2015).

22. Yamatsugu, K. *et al.* Kinetic analyses and structure-activity relationship studies of synthetic lysine acetylation catalysts. *Bioorg. Med. Chem.* **26**, 5359–5367 (2018).

23. Tamura, T. *et al.* Affinity-Guided Oxime Chemistry for Selective Protein Acylation in Live Tissue Systems. *J. Am. Chem. Soc.* **139**, 14181–14191 (2017).

24. Mizumoto, S. *et al.* Hydroxamic Acid‐Piperidine Conjugate is an Activated Catalyst for Lysine Acetylation under Physiological Conditions. *Chem. Asian J.* **15**, 833–839 (2020).

25. Fujimura, A. *et al.* Chemical catalyst/protein hybrid as artificial histone-modifying enzyme for epigenome manipulation. *ChemRxiv* (2022) doi:10.26434/chemrxiv-2022-xt610.

26. Jin, Q. *et al.* Distinct roles of GCN5/PCAF-mediated H3K9ac and CBP/p300-mediated H3K18/27ac in nuclear receptor transactivation. *EMBO J.* **30**, 249–262 (2011).

27. Evertts, A. G. *et al.* Quantitative Dynamics of the Link between Cellular Metabolism and Histone Acetylation. *J. Biol. Chem.* **288**, 12142–12151 (2013).

28. Simithy, J. *et al.* Characterization of histone acylations links chromatin modifications with metabolism. *Nat. Commun.* **8**, 1141 (2017).

29. Trefely, S. *et al.* Quantitative subcellular acyl-CoA analysis reveals distinct nuclear metabolism and isoleucine-dependent histone propionylation. *Mol. Cell* **82**, 447-462.e6 (2022).

30. Peng, C. *et al.* The First Identification of Lysine Malonylation Substrates and Its Regulatory Enzyme. *Mol. Cell. Proteomics* **10**, M111.012658 (2011).

31. Sabari, B. R., Zhang, D., Allis, C. D. & Zhao, Y. Metabolic regulation of gene expression through histone acylations. *Nat. Rev. Mol. Cell Biol.* **18**, 90–101 (2017).

32. Adamson, C., Kajino, H., Kawashima, S. A., Yamatsugu, K. & Kanai, M. Live-Cell Protein Modification by Boronate-Assisted Hydroxamic Acid Catalysis. *J. Am. Chem. Soc.* **143**, 14976–14980 (2021).

33. Sivanand, S., Viney, I. & Wellen, K. E. Spatiotemporal Control of Acetyl-CoA Metabolism in Chromatin Regulation. *Trends Biochem. Sci.* **43**, 61–74 (2018).

34. Liu, X. *et al.* Acetate Production from Glucose and Coupling to Mitochondrial Metabolism in Mammals. *Cell* **175**, 502-513.e13 (2018).

35. Baud, M. G. J. *et al.* Thioester derivatives of the natural product psammaplin A as potent histone

deacetylase inhibitors. *Beilstein J. Org. Chem.* **9**, 81–88 (2013).

36. Suzuki, T. *et al.* Thiol-based SAHA analogues as potent histone deacetylase inhibitors. *Bioorg. Med. Chem. Lett.* **14**, 3313–3317 (2004).

37. Ishiguro, T. *et al.* Synthetic Chromatin Acylation by an Artificial Catalyst System. *Chem* **2**, 840–859 (2017).

38. Kajino, H. *et al.* Synthetic hyperacetylation of nucleosomal histones. *RSC Chem. Biol.* **1**, 56–59 (2020).

39. Huang, H. *et al.* Lysine benzoylation is a histone mark regulated by SIRT2. *Nat. Commun.* **9**, 3374 (2018).

40. Jiang, Y. *et al.* Isonicotinylation is a histone mark induced by the anti-tuberculosis first-line drug isoniazid. *Nat. Commun.* **12**, 1–13 (2021).