JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY
Article
Cite This: J. Am. Chem. Soc. XXXX, XXX, XXX−XXX
pubs.acs.org/JACS

Cysteine-Aminoethylation-Assisted Chemical Ubiquitination of Recombinant Histones

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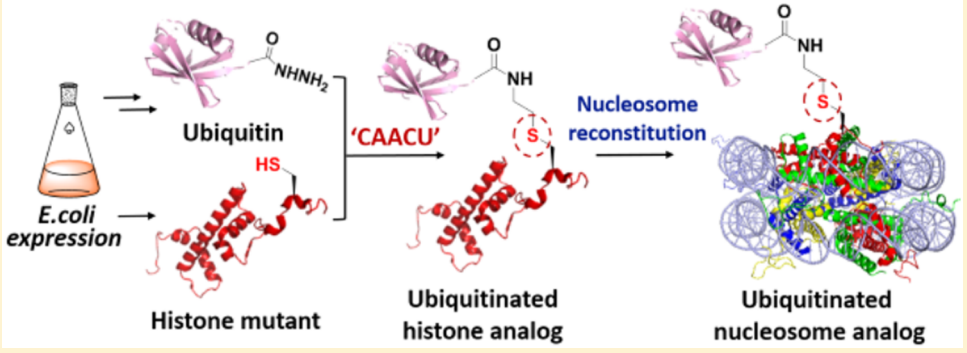
Supporting Information



ABSTRACT: Histone ubiquitination affects the structure and function of nucleosomes through tightly regulated dynamic reversible processes. The efficient preparation of ubiquitinated histones and their analogs is important for biochemical and biophysical studies on histone ubiquitination. Here, we report the CAACU (cysteine-aminoethylation assisted chemical ubiquitination) strategy for the efficient synthesis of ubiquitinated histone analogs. The key step in the CAACU strategy is the installation of an N-alkylated 2-bromoethylamine derivative into a recombinant histone through cysteine aminoethylation, followed by native chemical ligation assisted by Seitz's auxiliary to produce mono- and diubiquitin (Ub) and small ubiquitin-like modifier (SUMO) modified histone analogs. This approach enables the rapid production of modified histones from recombinant proteins at about 1.5−6 mg/L expression. The thioether-containing isopeptide bonds in the products are chemically stable and bear only one atomic substitution in the structure, compared to their native counterparts. The ubiquitinated histone analogs prepared by CAACU can be readily reconstituted into nucleosomes and selectively recognized by relevant interacting proteins. The thioether-containing isopeptide bonds can also be recognized and hydrolyzed by deubiquitinases (DUBs). Cryo-electron microscopy (cryo-EM) of the nucleosome containing H2BK$_C$34Ub indicated that the obtained CAACU histones were of good quality for structural studies. Collectively, this work exemplifies the utility of the CAACU strategy for the simple and efficient production of homogeneous ubiquitinated and SUMOylated histones for biochemical and biophysical studies.

INTRODUCTION

Histone post-translational modifications (PTM) such as acetylation, methylation, and ubiquitination are essential in the dynamic regulation of chromatin, which has been described in organisms ranging from yeast to humans.[1−3] Two types of protein modifiers, Ub and SUMO, can be installed onto the specific Lys sites of histones to form isopeptide bonds under ATP-dependent activation by a cascade of E1/E2/E3 enzymes.[4−6] It has been shown that ubiquitination or SUMOylation can affect the stability of the nucleosome and regulate transcriptional activation, DNA replication, and DNA-damage repair.[7−9] Such regulation is achieved through the recognition or processing of ubiquitinated or SUMOylated

Received: December 10, 2018

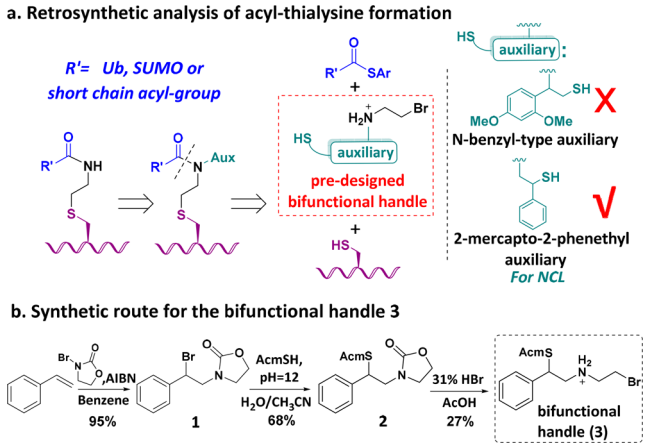
DOI: 10.1021/jacs.8b13213
J. Am. Chem. Soc. XXXX, XXX, XXX−XXX

nucleosomes by different interacting proteins. For example, a nucleosome containing both Lys15-ubiquitinated histone H2A (H2AK15Ub) and Lys20-dimethylated histone H4 (H4K20me2) can recruit p53-binding protein (53BP1) during the process of DNA damage repair, thereby triggering the downstream repair process.[10] Insights into the structure and functional regulation of nucleosomes can be inferred by the elucidation of the molecular mechanisms by which ubiquitinated or SUMOylated histones interact with proteins,[11,12] but such studies are predicated upon the availability of reasonable amounts of site-specifically ubiquitinated or SUMOylated histones.[7,11]

The enzymatic synthesis of ubiquitinated or SUMOylated histones remains difficult because the enzyme responsible for a given modified histone may be unknown or difficult to isolate in an active form.[9,13] Total chemical protein synthesis[14,15] can also be used to access ubiquitinated and SUMOylated histones[16−21] but is technically too difficult for the majority of biochemistry laboratories to carry out. Meanwhile, although semiprotein synthesis from expressed histone segments avoids lengthy peptide synthesis, this technology is often restricted to ubiquitination and SUMOylation close to N or C termini.[9,22−25] For these reasons, strategies that take advantage of the unique reactivity of the nucleophilic Cys sulfhydryl group[26−31] are more often used for the site-specific conjugation of Ub to recombinant full-length histones. These strategies are mostly based on the construction of isopeptide bond analogs at the selected Cys through a disulfide-, hydrazide-, or 1,3-dichloroacetone (DCA)-derived linkage.[13,30−34] In terms of application, Wolberger and Brik et al. prepared a DCA-derived H2BK120Ub analog and incorporated it into NCPs (nucleosome core particle) to generate a crystal structure with the Ubp8/Sgf11/Sus1/Sgf73 DUB module.[35] Muir et al. prepared a disulfide-based H2BK120Ub-containing nucleosome bearing site-specifically installed photo-cross-linkers and used it to study the activation mechanism of histone H3 methyltransferase hDot1L.[33] Despite these advances, the labile nature of the disulfide limits its use under reducing conditions, and DCA or hydrazide mimics may not adequately imitate the native isopeptide bond.[13,36] Therefore, we wished to examine whether there is an alternative Cys-based method for the synthesis of ubiquitinated or SUMOylated histone analogs based upon a more stable and more structurally similar isopeptide bond mimic.

Here, we report a cysteine-aminoethylation-assisted chemical ubiquitination (CAACU) strategy for making ubiquitinated or SUMOylated histone analogs from recombinant proteins. The key step of CAACU is the site-specific installation of an N-alkylated 2-bromoethylamine derivative onto the Cys residue of recombinant histones (Scheme 1a). Ubiquitination and even SUMOylation can then be accomplished through auxiliary (Aux)-mediated native chemical ligation (NCL) with recombinant Ub or SUMO hydrazides. Because both histones and Ub (or SUMO) are readily acquired through recombinant expression, the use of CAACU enables multimilligram quantities of homogeneous mono- and di-Ub, and SUMO-modified histone analogs are rapidly obtained. The resulting ubiquitinated or SUMOylated histone analogs are chemically stable and differ from the native isopeptide bond by only one atom, and their utility for biochemical and biophysical studies on histone and nucleosome ubiquitination and SUMOylation is established herein.

Scheme 1. Combination of Cys Aminoethylation with Auxiliary-Mediated NCL[a]



[a](a) Retrosynthetic analysis of acyl-thialysine formation. The key reagent in the strategy is the pre-designed bifunctional handle that bears two reactive units: an N-alkylated 2-bromoethyl amine scaffold, along with an auxiliary group allowing the NCL. (b) Synthesis of **3** through three steps in 18% overall yield.

RESULTS AND DISCUSSION

Cys Aminoethylation with a Bifunctional Handle. The Cys aminoethylation reaction was originally used by Lindley in 1956 to convert a Cys residue in a protein to a Lys-mimicking structure.[37] In 2007, Shokat et al. reported an important and hitherto widely used application of this chemistry for the production of methylated histone analogs.[38] Nonetheless, Cys aminoethylation cannot be directly used to acquire proteins bearing acyl-Lys-type modifications (Scheme S1a). Here, we envisaged that a 2-bromoethylamine derivative carrying a removable NCL-auxiliary would enable the generation of the acyl aminoethylcysteine structure (Scheme 1a). However, our initial attempts to synthesize a 2-bromoethylamine derivate containing the commonly used acid-cleavable N-benzyl-type auxiliary[39] failed because β-bromination of an amine bearing an acid-sensitive benzyl group proved very difficult (Schemes S1b).

Fortunately, Seitz et al. recently invented a new type of removable auxiliary bearing a 2-mercapto-2-phenethyl group.[40] We hypothesized that the absence of α-branching at the central amino group of this auxiliary might allow the required β-bromination under acidic conditions. With this in mind, we converted styrene to compound **1** in 95% yield via the AIBN (2,2′-azobis(isobutyronitrile))-promoted addition of 3-bromo-2-oxazolidinone. AcmSH was then used to displace the bromine in **1** to produce compound **2** in 68% yield. Finally, the ring opening of the oxazolidinone moiety of **2** was carried out in HOAc containing 31% HBr[41] to afford the desired 2-bromoethylamine derivative in 27% yield (Scheme 1b). Thus, key bifunctional handle **3** can be rapidly prepared in three steps in an overall yield of 18%, which bodes well for its future application.

Next, we used a Cys-containing model peptide to examine the aminoethylation of **3** under different conditions (Figures S5 and S6). At pH 8.0−8.5, the aminoethylation reaction proceeded at high rates in HEPES buffer, converting the model peptide to the desired modification product in yields of 85−90% (by HPLC) within 5 h. We then examined the efficiency of the aminoethylation reaction at different concentrations of **3**

DOI: 10.1021/jacs.8b13213
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from 10 to 60 mM. It was found that although the reaction was faster at 40−60 mM **3**, bis-alkylated side products were observed under these conditions (Figure S6). Thus, to generate the desired product in good homogeneity, the concentration of **3** was maintained at 20−30 mM.

The optimized conditions were then applied to full-length histones. Because only one Cys is present in the four core histones (Cys110 in H3), histones are particularly amenable to Cys-based conjugation.[13,31] We first tested the Cys aminoethylation of **3** with the histone H2B mutant containing a Cys at position 34 (H2BK34C, **4**). To obtain **4**, the gene of *Xenopus laevis* histone H2B containing the K34C mutation was cloned into the pET15 vector and expressed in *E. coli*.[42] After purification from the inclusion body, H2BK34C was obtained in a 50−60 mg/L LB medium. For the reaction between **3** and H2BK34C, aqueous buffer containing 6 M guanidine hydrochloride (Gn•HCl) was used. Tris(2-carboxyethyl) phosphine (TCEP) and free methionine were added to maintain the reducing conditions and avoid oxidation of the thioether bond. The reaction was performed at pH 8.5 with 20 mM **3** for 5 h. HPLC analysis showed that the conversion of H2BK34C was 50%, even after the reaction was extended to 10 h. A key finding was that the addition of a second batch of **3** (20 mM) and TCEP to the reaction mixture after 5 h could greatly improve conversion, and ca. 85% of desired product **5** was obtained in a total of ca. 9 h (Figure 1a). The same protocol

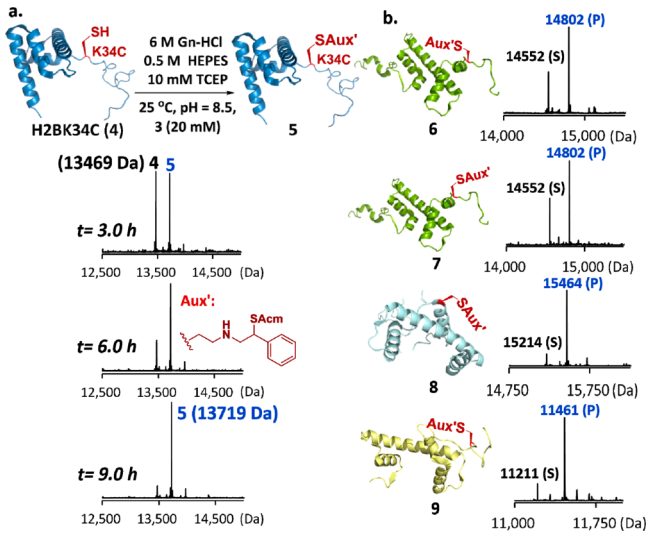

Figure 1. Incorporation of **3** into histones. (a) Installing **3** into H2BK34C under the following reaction conditions: protein (10 mg/mL), 6 M Gn-HCl, 0.5 M HEPES, 10 mM TCEP, 20 mM **3**, 37 °C, pH 8.5. After 5 h, additional 20 mM **3** and 10 mM TCEP were supplied, and ESI-TOF MS monitoring showed that the conversion of H2BK34C exceeded 80% after 9 h. (b) ESI-TOF MS showed that **3** could be cleanly installed in different histones. Starting materials are labeled "S", and species corresponding to the correct product mass are labeled "P".

was also tested for other histones (H2AK13C, H2AK15C, H3K56C, and H4K12C) to form **6**, **7**, **8**, and **9**. To our delight, all reactions proceeded smoothly with conversions ranging from 60 to 85% (Figure 1b).

Synthesis of Ubiquitinated and SUMOylated Histone Analogs. After validating the efficient Cys aminoethylation reaction of **3**, we turned to the synthesis of the ubiquitinated histone analogs through auxiliary-mediated NCL. H2B bearing Ub at K34, a PTM recently found to play an important role in regulating gene transcription, was examined first.[43,44] The synthesis route of H2BK$_C$34Ub is shown in Figure 2a. A key substrate required for the synthesis was Ub(1-76)-Mesna or its hydrazinolysis product Ub(1-76)-NHNH$_2$ (**11**), which could be produced in 4 to 5 mg/L of LB medium through intein technology.[45] To improve the efficiency of the synthesis of **11**, we devised a new approach wherein the hydrazinolysis of Ub variant Ub(1-77D) was accomplished using Ub C-terminal hydrolase YUH1.[46] Thus, Ub(1-77D) was expressed (>50 mg/L LB medium) first and was then treated with aqueous hydrazine (5% v/v) in the presence of YUH1 (1 μM) at pH 7.4 and 0 °C. This led to the formation of **11** (80% conversion) within 2 h in a yield of 30−40 mg from 1 L expression.

With **11** in hand, we removed the Acm protecting group of **5** using AgOAc or PdCl$_2$ to produce **10**.[47,48] After purification, **10** was ligated with **11** using a hydrazide-based auxiliary-assisted NCL[49,50] at pH 6.9 to generate **12** within 6 h (44% isolated yield). Note that the conversion of **10** to **12** was observed to be almost quantitative by HPLC reaction monitoring, a surprising result given that use of the common N-benzyl-type auxiliary often leads to significant hydrolysis during ligation. This finding exemplifies the advantage of Seitz's new auxiliary—the absence of α-branching at the central amino group.[51] Finally, the auxiliary group on **12** was readily removed within 10 h in 85% conversion (Figure 2c).[40] After purification and lyophilization, we obtained H2BK$_C$34Ub (**13**) in an isolated yield of 48%. The identity and purity of **13** were ascertained by HPLC, ESI-MS, and SDS-PAGE analysis (Figure 2b,h). MS-MS analysis further confirmed that Ub was installed at the correct site (Figure 2d).

The above results demonstrate the effectiveness of CAACU for the synthesis of ubiquitinated histones in just four steps: Cys aminoethylation, Acm deprotection, NCL, and auxiliary removal. In a typical experiment starting with the expression of H2BK34C from 1 L of LB medium, we could generate ca. 6 mg of H2BK$_C$34Ub in an overall yield of 12% in ca. 2 weeks (including reaction, separation, and lyophilization). Using the same strategy, we could readily conjugate Ub onto the designed sites of other core histones. For instance, we successfully synthesized H2AK$_C$119Ub (1.5−2 mg from 1 L expression of H2AK119C), which was reported to mediate DNA-damage-induced local transcriptional repression.[52] We also obtained H2AK$_C$13Ub and H2AK$_C$15Ub (3 to 4 mg from 1 L expression of H2AK13C or H2AK15C), which correspond to the ubiquitination at the N-terminal tail of histone H2A and play important roles in DNA-damage repair.[53] Moreover, we tested CAACU for the SUMOylation of histone H4, a modification that was recently reported to be involved in the modulation of chromatin compaction and the methylation state.[54] SUMO3-intein fusion protein was expressed with the Cys47Ser mutation. Treatment of this intein fusion protein with aqueous hydrazine (5% v/v) led to the formation of SUMO3-(2-92)-C47S-NHNH$_2$ (12−15 mg/L of LB medium, Figure S9d). This SUMO hydrazide could be readily ligated with H4 modified with an auxiliary at position 12, leading to the formation of H4K$_C$12SUMO3 (2 mg from 1 L expression of H4K12C). This SUMOylated histone was successfully characterized by HPLC, high-resolution ESI-MS, and SDS-PAGE (Figure 2e,h).

Finally, we wished to show that the CAACU strategy could also be used to make oligo-Ub analogs and histones modified

DOI: 10.1021/jacs.8b13213
J. Am. Chem. Soc. XXXX, XXX, XXX−XXX

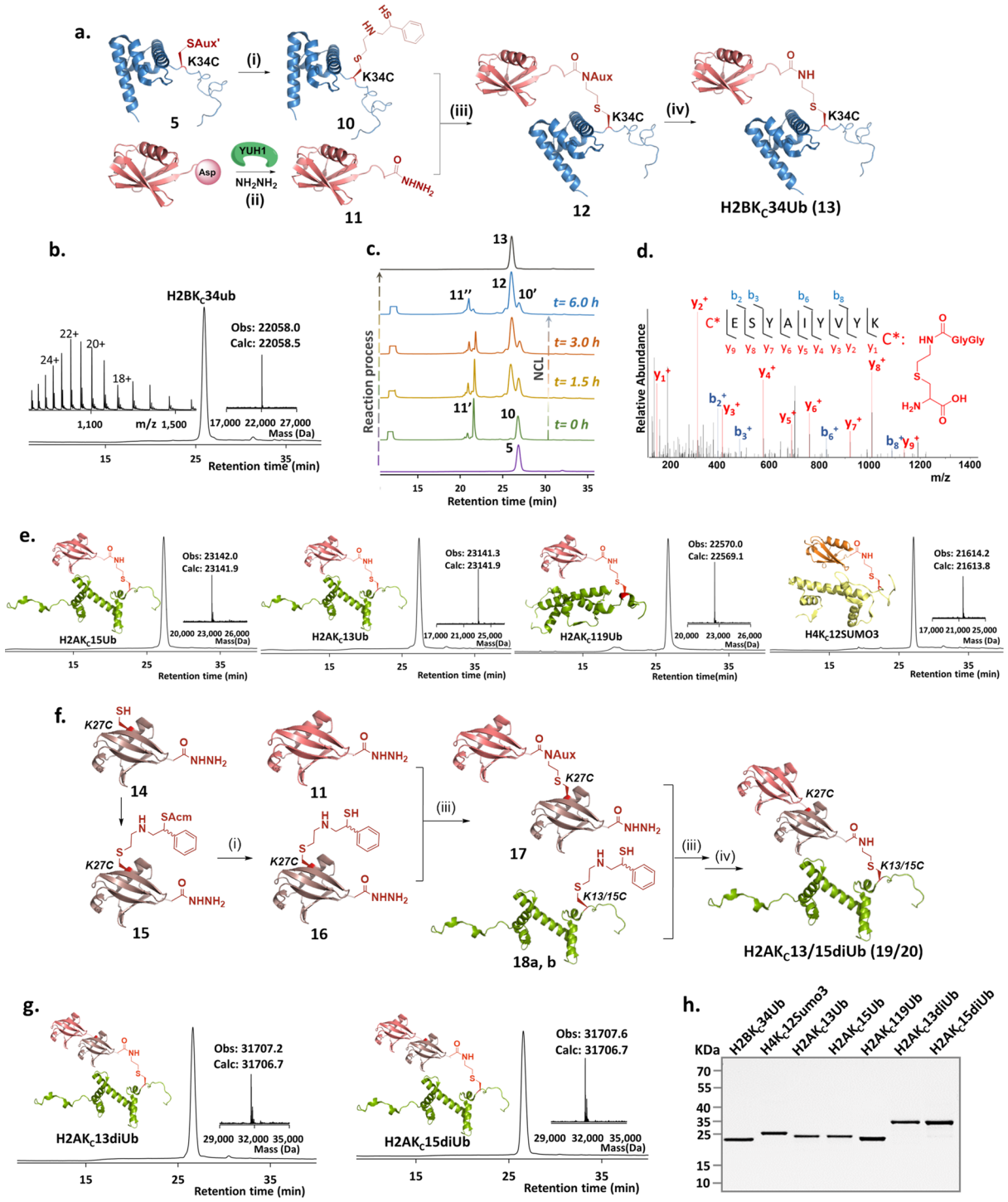

Figure 2. Semisynthesis of Ub- and SUMO-modified histone analogs through the CAACU strategy. (a) The synthesis route of $H2BK_C34Ub$. Reaction conditions: (i) **5** (0.5 mM, 1.0 equiv), AgOAc (15 mM, 30 equiv), H_2O/CH_3COOH = 1:1 (v/v), 37 °C, 12 h. (ii) Ub(1-77D) (15−20 mg/mL), YUH1 (1.0 μM), 50 mM Tris buffer containing 5% (v/v) NH_2NH_2, pH 7.4, 0 °C, 1 h. (iii) **10** (0.5 mM, 1.0 equiv) was ligated with **11** (0.75 mM, 1.5 equiv) through hydrazide-based NCL. (iv) Removing the auxiliary group of **12** (2 to 3 mg/mL) was carried out in denatured buffer containing 6.0 M Gn-HCl, TCEP (400 mM), morpholine (2.0 M), pH 8.5, 37 °C, 12 h. (b) Analytical RP-HPLC (214 nm) and high-resolution ESI-MS (average isotope) characterizations of purified **13**. (c) HPLC traces (214 nm) for the synthesis of $H2BK_C34Ub$. The NCL between **10** and **11′** (Ub(1-76)-MPAA thioester) was monitored at 0, 1.5, 3.0, and 6.0 h, respectively. **10′** and **11′′** indicate other byproducts and Ub(1-76)-COOH, respectively. (d) MS-MS analysis of $H2BK_C34Ub$ demonstrated the installation of the Ub unit at the correct position. (e) Analytical RP-HPLC (214 nm) and deconvoluted mass of purified $H4K_C12SUMO3$, $H2AK_C13/15Ub$, and $H2AK_C119Ub$. (f) Synthesis route of Lys27-linked diubiquitinated H2A ($H2AK_C13/15\text{-}K_C27diUb$, **19**, **20**). (g) Analytical RP-HPLC (214 nm) and deconvoluted mass of purified **19** and **20**. (h) SDS-PAGE analysis of the semisynthetic Ub- and SUMO-modified histone analogs.

with oligo-Ubs. To give an example, we chose H2A carrying Lys27-linked di-Ub on Lys13 or 15, which was previously reported to play roles in 53BP1-mediated DNA damage repair (Figure 2f).[55,56] First, UbK27C-$NHNH_2$ (**14**) was reacted

DOI: 10.1021/jacs.8b13213
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smoothly with **3** through Cys aminoethylation to generate **15** (ca. 85% HPLC conversion, Figure S10b). After the removal of Acm from **15**, product **16** was successfully ligated with Ub(1-76)-$NHNH_2$ (**11**) through hydrazide-based ligation in 5 h (isolated yield = 51%, Figure S10c). In this fashion, the desired di-Ub K_C27diUb-$NHNH_2$ (**17**) was obtained, which was then ligated with H2A bearing an auxiliary at position 13 or 15. After the removal of the auxiliary, H2AK_C13-K_C27diUB (**19**) or H2AK_C15-K_C27diUB (**20**) was successfully obtained (ca. 3 mg from 1 L expression of H2AK13C or H2AK15C) and fully characterized by HPLC, ESI-MS, and SDS-PAGE (Figure 2g,h). Two conclusions can be drawn from the above experiments: First, through the use of K-to-C mutated Ub hydrazide, different di- and, in principle, oligo-Ub can be conveniently obtained from recombinant materials. Second, oligo-Ub hydrazide can also be used in CAACU, leading to substrate proteins modified with oligo-Ub at a predesignated position. Studies toward these targets are ongoing in our laboratory.

Nucleosome Reconstitution and Reader Recognition. The reconstitution of ubiquitinated or SUMOylated nucleosomes enables studies on their interactions with reader proteins. Here, we examined the in vitro reconstitution of NCPs with the synthetic ubiquitinated histone analogs from the CAACU strategy. First, H2BK_C34Ub, H2AK_C13Ub, or H2AK_C15Ub was individually incorporated into octamers together with other core histones and purified by size-exclusion chromatography (Figure S13). Then the octamer was reconstituted into nucleosomes with a 147 base pair 601 DNA sequence through stepwise dialysis. SDS-PAGE and native-gel analyses confirmed that synthetic ubiquitinated histones were stoichiometrically incorporated into the nucleosomes (Figure 3a,b).[42]

Next, we tested the selective recognition properties of the ubiquitinated nucleosomes with the interacting proteins (readers). 53BP1 is a critical regulator of the cellular response to DNA double-strand breaks and was recently discovered to bind specifically to NCPs containing both H4-K20me2 and H2AK15Ub but not H2AK13Ub.[10,55] Here we used NCPs containing H2AK_C13Ub or H2AK_C15Ub to do the test and performed pull-down assays with GST-tagged 53BP1 consisting of the tandem Tudor-UDR segment (GST-Tudor-UDR, residues 1484−1631) as previously described.[55] H2AK_C13Ub or H2AK_C15Ub was incorporated into NCPs together with H4K_C20me2 (made by Shokat's strategy).[38] The resulting NCPs as well as an NCP containing wild-type H2AK13Ub (H2AK13Ub_{wt}) as a control were incubated with GST-Tudor-UDR. After the removal of nonspecific binding proteins by thorough washing of the glutathione-sepharose resin, the samples were resuspended directly in protein loading buffer and then analyzed by SDS-PAGE. It was found that 53BP1 Tudor-UDR protein selectively bound to NCP containing H2AK_C15Ub but not to NCP containing H2AK_C13Ub or H2AK13Ub_{wt} (Figure 3c). The pull-down experiments were also repeated and analyzed by immunoblotting using an anti-H2A specific antibody, with the same result (Figure 3d). In addition, size exclusion chromatography and SDS-PAGE analysis further confirmed that NCP containing H2AK_C15Ub could form a stable complex with 53BP1 (Figure 3e,f). These results suggest that NCP containing the ubiquitinated histones prepared by CAACU exhibit similar molecular recognition properties to their natural counterparts.

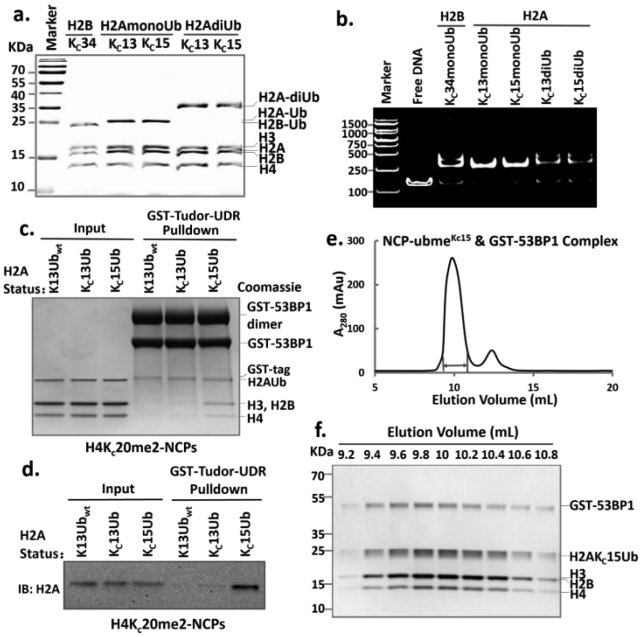

Figure 3. Nucleosome reconstitution and selective recognition of the interacting protein. (a) SDS-PAGE analysis of reconstituted histone octamers containing ubiquitinated H2B and H2A. (b) Sybr Gold-stained native gels of reconstituted NCPs containing ubiquitinated H2B and H2A. (c and d) GST-Tudor-UDR pull-down assays of NCPs containing ubiquitinated H2A analogs and H4K_C20me2 in which H2AK13Ub_{wt}-containing NCP were used as the control [(c) Coomassie-stained and (d) immunoblotting anti-H2A]. (e) Gel filtration chromatography of the preformed NCP-$Ubme^{K15}$/GST-53BP1 complex. (λ = 280 nm, Superdex 200 Increase 10/300 GL). (f) SDS-PAGE analysis of the peak fraction of the NCP-$ubme^{K15}$/GST-53BP1 complex, stained with Coomassie blue, in which the elution volume of each sample was labeled.

Sulfur-Containing Isopeptide Bonds Can Be Recognized and Hydrolyzed by DUBs. Histone deubiquitination catalyzed by various DUBs occurs in the processes of DNA damage repair, gene activation inhibition, and chromosome condensation,[57] and its dysfunction is implicated in many pathological processes such as cancer, aging, and infertility.[58] Site-specifically ubiquitinated histones and nucleosomes are needed for biochemical and screening studies of histone DUBs, but analogs bearing 1,3-dichloroacetone moieties are not hydrolyzable substrates. Previously, Strieter et al. demonstrated that synthetic diUbs with thioether-based isopeptide bonds are functionally active substrates for DUBs.[59] We wished to examine whether ubiquitinated histones made by CAACU were also functionally active substrates for DUBs. To this end, we treated H2AK_C13Ub with two representative DUBs, namely, YUH1 and UCHL1.[60] For direct comparison, native H2AK13Ub was tested alongside H2AK_C13Ub synthesized by CAACU. SDS-PAGE monitoring showed that H2AK13Ub was rapidly hydrolyzed by YUH1 to Ub and H2A in 0.5 h. However, even when the incubation time was extended to 1.5 h, very little H2AK13Ub was hydrolyzed by UCHL1. These observations indicate that the two DUBs had very different reactivity toward H2AK13Ub, an interesting phenomena that was also observed for H2AK_C13Ub (Figure 4a, 4b). We also found that YUH1 can rapidly hydrolyze H2AK_C15Ub while UCHL1 cannot (Figure S16). Therefore, the thioether-containing isopeptide bond could be processed by DUBs to a similar extent as for a native isopeptide bond.

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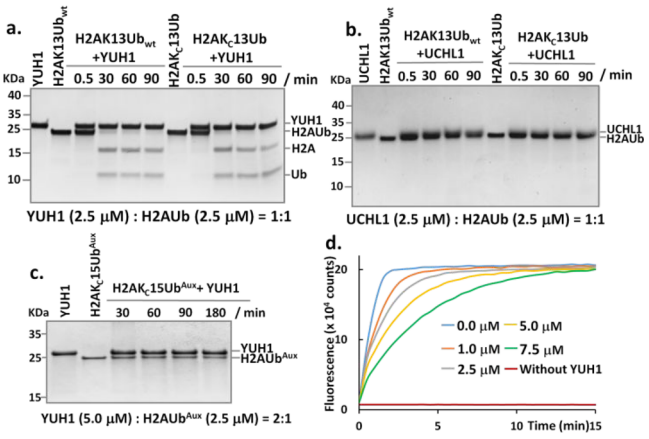

Figure 4. Ubiquitinated histones made through CAACU are functionally active substrates for DUBs. (a, b) DUB assays of YUH1 (2.5 μM) and UCHL1 (2.5 μM) toward H2AK13Ub$_{wt}$ (2.5 μM) and H2AK$_C$13Ub (2.5 μM). (c) DUB assay of YUH1 (5.0 μM) toward nonhydrolyzable H2AK$_C$15Ub^{Aux} (2.5 μM). (d) Non-hydrolyzable H2AK$_C$15Ub^{Aux} (0, 1.0, 2.5, 5.0, and 7.5 μM) acts as a competitive inhibitor of Ub-AMC (2.0 μM) cleavage by YUH1 (10 nM). Excitation wavelength = 360 nm and emission wavelength = 460 nm.

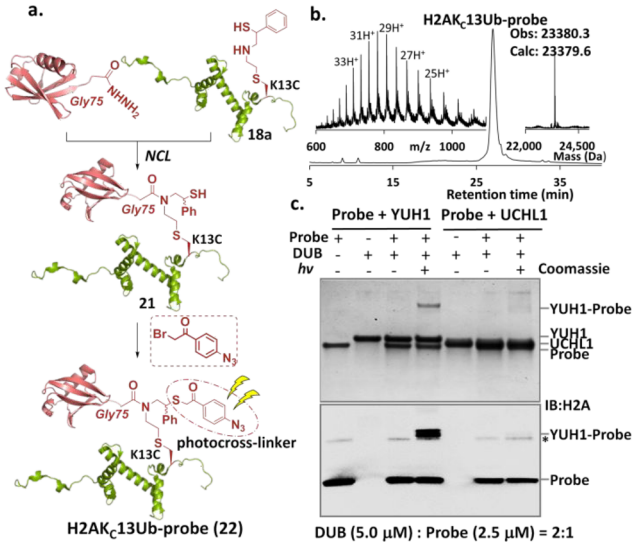

Figure 5. H2AK$_C$13Ub photoaffinity probe. (a) Synthesis of probe **22**. The aryl azide photo-cross-linker was installed at the position close to the isopeptide bond. (b) Analytical RP-HPLC (λ = 214 nm), ESI-MS, and deconvoluted mass of purified H2AK$_C$13Ub probe. (c) SDS-PAGE and Western blot analysis (anti-H2A antibody) of the photo-cross-linking reaction between DUBs (YUH1 or UCHL1, 5.0 μM) and the H2AK$_C$13Ub probe (2.5 μM) in the presence or absence of ultraviolet light irradiation (λ = 365 nm). * indicates the self-cross-linked product of the probe.

Meanwhile, we tested the hydrolytic activity of YUH1 on H2AK$_C$15Ub that still carried the auxiliary group at its isopeptide bond (H2AK$_C$15Ub^{Aux}). H2AK$_C$15Ub^{Aux} was found to be almost impervious to hydrolysis by YUH1 (Figure 4c), consistent with the previous finding that the N-alkylated isopeptide bond is resistant to DUBs.[61] Nonetheless, our fluorescence assay showed that H2AK$_C$15Ub^{Aux} was a competitive inhibitor of YUH1 during its cleavage of Ub-AMC (Ub-7-amino-4-methylcoumarin). This finding suggests that binding takes place between H2AK$_C$15Ub^{Aux} and YUH1 (Figure 4d) and that the development of profiling tools based on photo-cross-linking might be possible.[62,63]

To test this idea, we introduced an aryl azide photo-cross-linker at the auxiliary of H2AK$_C$13Ub to construct a photoaffinity probe (**22**, Figure 5a,b). Probe **22** was incubated with YUH1 and UCHL1 at 0 °C for 1 h and then exposed to 365 nm UV light for 5 min. An analysis of the reaction mixture by SDS-PAGE and immunoblotting revealed the expected cross-linked band of probe **22** and YUH1 at the correct molecular weight position. This band was attributed to covalent cross-linking because no band was detected at this position in the absence of UV irradiation. In contrast, **22** did not cross-link with UCHL1 (Figure 5c), consistent with the aforementioned observation that UCHL1 was not active on H2AK13Ub. Collectively, our experiments suggest that the thioether-containing isopeptides in ubiquitinated histones made through CAACU were hydrolyzable by DUBs. Thus, the histone analogs made by CAACU may constitute functionally active reagents for the profiling and study of DUBs at the histone or nucleosome levels.

Cryo-EM Studies on a Ubiquitinated Nucleosome Containing H2BK$_C$34Ub. Structural studies of ubiquitinated nucleosomes and their complexes with many interacting proteins have yielded fundamental insight into the recognitory and regulatory mechanisms of chromatin ubiquitination.[10,35] An essential prerequisite for such studies is the ready availability of high-quality, homogeneous ubiquitinated NCP. The above results exemplify the utility of the CAACU strategy to enable facile access to multimilligram quantities of ubiquitinated or SUMOylated histone analogs. We sought to ascertain whether these synthetic proteins could also be used for structural studies on ubiquitinated nucleosomes through single-particle cryo-EM (cryo-EM).

Accordingly, we examined the nucleosome containing synthetic H2BK$_C$34Ub (i.e., H2BK$_C$34Ub-NCP) by cryo-EM. H2BK$_C$34Ub-NCP was reconstituted, and purified samples of it were flash frozen. Single-particle cryo-EM pictures were recorded using a Tecnai F20 electron microscope operated at 200 kV equipped with a Falcon-3 direct electron detector. After 2D/3D classification and refinement, we obtained three different types (types I–III) of H2BK$_C$34Ub-NCP cryo-EM structures at average resolutions of 7.1, 10.0, and 11.7 Å, respectively (Figure S18c and Figure S20). Surface views of these three structures show them to be disklike and consistent with classic NCP structures[64,65] and bear extra density attributable to Ub on the side of NCP. The density attributed to Ub is significantly different among the three structures, consistent with a previous theory that the Ub domains in ubiquitinated nucleosomes are structurally mobile.[10,35,66]

In the type-I structure, the DNA duplex and core secondary elements of the histone octamers fit unambiguously onto the cyro-EM map, with only one protrusion corresponding to the Ub linked to the upper K34 residue (Figure 6a and Figure S19). The density for the Ub moiety was lower than for the rest of the structure, and the local resolution of the protruding part was lower than that of NCP (Figure S18e). Nevertheless, we could locate the protruding density adjacent to the K34 of H2B. Furthermore, the DNA densities adjacent to the K34 of H2B in the cryo-EM map deviated from those in the native NCP structure, causing an expansion of the distance between the two DNA duplexes within H2BK$_C$34Ub-NCP by ca. 2 Å (Figure 6b). The resulting space was large enough to allow the

DOI: 10.1021/jacs.8b13213
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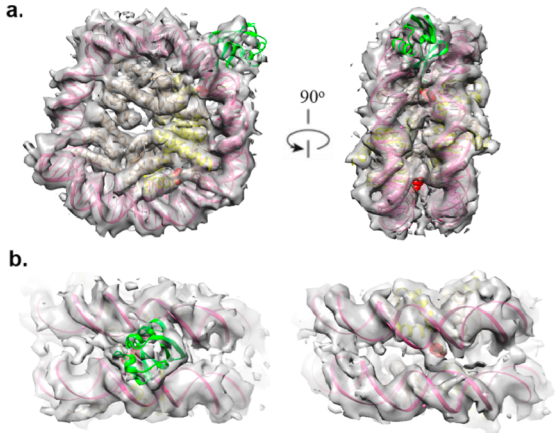

Figure 6. Cryo-EM structure of type-I H2BK$_C$34Ub NCP. (a) Surface of type-I H2BK$_C$34Ub NCP viewed along the DNA axis and the orthogonal direction. Rigid-body fitting of the nucleosome crystal structure (DNA, PDB code 1KX5; octameric histones, PDB code 2CV5) into H2BK$_C$34Ub NCP density is shown as ribbons (purple, DNA; yellow, histone H2BK34C; red, K34 of H2B). Ub (green color) could not be readily placed in the attributed density. (b) DNA densities in the map deviate from those in the nucleosome crystal structure.

Ub modifier to pass through the DNA duplexes. Secondary elements of the type II and III structures could not be assigned because of their poor resolution, but the protruding densities adjacent to the lower K34 (type II) or upper and lower K34 (type III) were apparent (Figure S21). The Ub domain could not be readily identified in the attributed density because of the structural flexibility. Note that the cryo-EM structures of H2BK$_C$34Ub-NCP solved here were of a higher resolution but otherwise were almost identical to that of their previously reported, native counterpart obtained in a laborious total synthesis.[42] We thus anticipate that the ubiquitinated histone analogs prepared by CAACU would be suitable for structural studies on nucleosome modifications.

Short-Chain Lys Acylation. Apart from histone ubiquitination and SUMOylation, a number of interesting short-chain Lys acylations (e.g., β-hydroxybutyrylation and succinylation) have been mapped on histones.[67] The functional and structural roles of many of these Lys acylation modifications are inadequately characterized, in part because of the difficulty in obtaining site-specifically acylated histones or analogs. Although methods such as thiol−ene ligation, Staudinger ligation, and hydrazide mimics have been developed, new and complementary strategies will improve the capability of the tool box.[30,68,69]

To demonstrate that the central concept of CAACU (i.e., a combination of Cys aminoethylation and protein ligation) is also suitable for the synthesis of short-chain Lys acylated histones, we sought to synthesize an analog of H2B bearing Lys β-hydroxybutyrylation at K34 (i.e., H2BK$_C$34Bhb). This new type of epigenetic regulatory marker was recently identified by proteomics and proposed to be involved in regulating gene expression.[70] However, an in vitro preparation of the full-length histone bearing Lys β-hydroxybutyrylation or an analog has yet to be reported. In our experiment, we used the Bhb-thioester, obtained in one step from (*R*)-3-hydroxybutyric acid in 58% yield, to ligate with the auxiliary-containing H2BK34C derivative (**10**) (Figure 7a). After removal of the auxiliary, desired product H2BK$_C$34-Bhb was

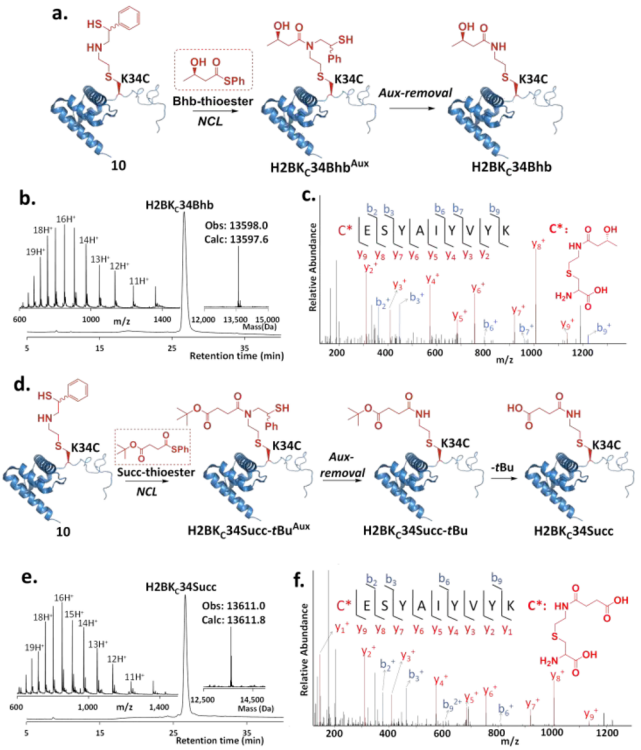

Figure 7. Site-specific incorporation of short-chain Lys acylation analogs into histones. (a and d) Strategy for the synthesis of H2BK$_C$34Bhb and H2BK$_C$34Succ. (b and e) Analytical RP-HPLC chromatogram (λ = 214 nm) and deconvoluted mass of purified products. (c and f) Tandem MS analysis of the trypsinized K$_C$Bhb- and K$_C$Succ-containing peptide segments.

obtained in 4 to 5 mg from 1 L expression of H2BK34C (Figure 7b). MS-MS analysis revealed a peptide segment containing H2BK$_C$34Bhb, which confirmed β-hydroxyisobutyrylation at the K34 residue (Figure 7c).

In addition, we synthesized the succinylated H2B (H2BK$_C$34Succ) analog using the same concept. To avoid intramolecular cyclization, the carboxylate was protected with a *tert*-butyl protecting group (Figure 7d). The desired product was fully characterized by HPLC, ESI-MS, and MS-MS (Figure 7e,f). Thus, the CAACU strategy also amounts to a general method of installing short-chain Lys acylation onto histones.

SUMMARY

Biochemical analyses of the effects of ubiquitination and SUMOylation on the structures and functions of nucleosomes and their complexes are necessary to understand the regulation of Ub and SUMO modification on transcriptional activation, DNA replication, and DNA-damage repair. To more easily and cheaply obtain the requisite modified protein reagents, we developed the CAACU strategy that combines the powers of Cys aminoethylation and protein ligation and established it as an easy-to-implement method for obtaining multimilligram quantities of ubiquitinated and SUMOylated histones from recombinantly expressed proteins. The products yielded by this strategy are chemically stable and exhibit only minor structural and therefore functional perturbation of the isopeptide bond. By testing the molecular recognition of reader proteins, hydrolytic processing by deubiquitination enzymes, and cryo-EM structural determination, we demonstrated that the ubiquitinated and SUMOylated histones made by CAACU

DOI: 10.1021/jacs.8b13213
J. Am. Chem. Soc. XXXX, XXX, XXX−XXX

are applicable to biochemical and biophysical studies. Finally, the CAACU strategy was used to synthesize di-Ub modified histones and histones bearing Lys succinylation and β-hydroxybutyrylation, further broadening its scope. Accordingly, we anticipate that the concept of combining Cys aminoethylation and protein ligation may enable the development of more synthetic methodologies applicable to studies of protein post-translational modifications.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.8b13213.

Experimental details and compound characterization; general methods; organic synthesis; protein expression and purification; octamer and nucleosome reconstitution and purification; NCP pull-down assays; NCP-ubme^{Kc15} and GST-53BP1 complex formation; preparation of protein hydrazide; semisynthesis of the Ub- and SUMO-modified histone analogs, H2AK$_C$13Ub photoaffinity probe, and H2BK$_C$34Bhb and H2BK$_C$4Succ through the CAACU strategy; and deubiquitination, inhibition, and photo-cross-linking assays (PDF)

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This study was supported by the National Key R&D Program of China (2017YFA0505200 and 2017YFA0505400) and the NSFC (91753205, 21532004, 21877024, 81621002, 21621003 and 21708036). We thank the protein chemistry facility at the Center for Biomedical Analysis of Tsinghua University for sample analysis. We thank the staff at the Center for Integrative Imaging (University of Science and Technology of China) for their assistance during cryo-EM data collection.

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