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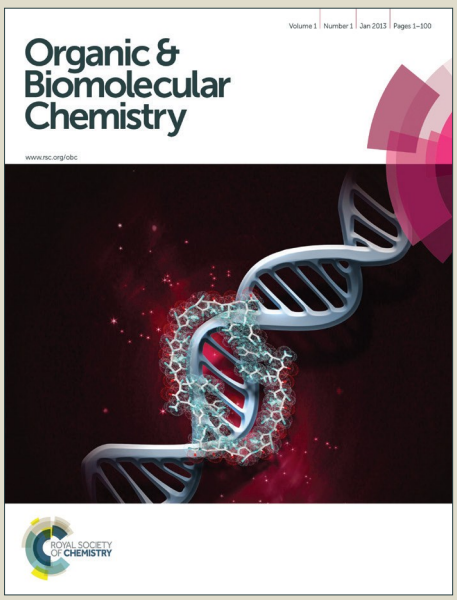





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# Semisynthetic UbH2A Reveals Different Activities of Deubiquitinases and Inhibitory Effects of H2A K119 Ubiquitination on H3K36 Methylation in Mononucleosomes †

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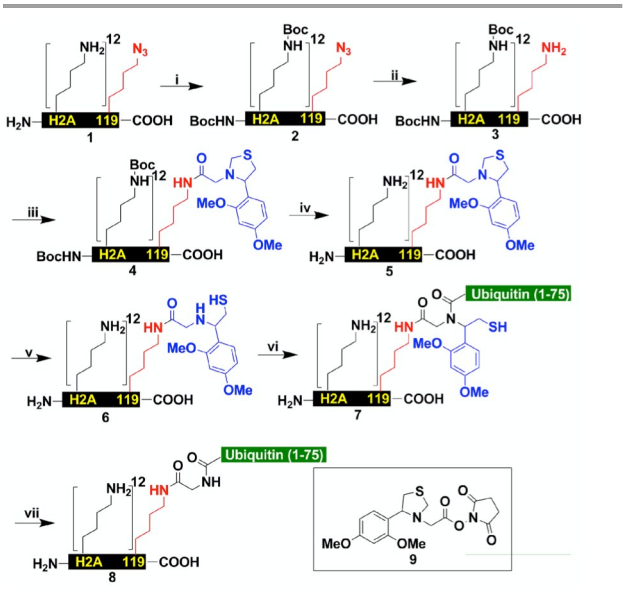
**Using a genetically incorporated azidonorleucine for ubiquitin installation, we prepared multi-milligram quantities of H2AK119ub (ubH2A). With a native isopeptide linkage, the synthetic ubH2A was used to study the activity of deubiquitinases and crosstalk between H2A ubiquitination and H3K36 methylation in the context of chemically defined mononucleosomes.**

Post-translational modification (PTM) is a fundamental cellular mechanism to modulate protein function.[1] One prominent example is the histones, which undergo various PTMs, including acetylation, methylation, phosphorylation and ubiquitination.[2] These modifications regulate various DNA-dependent processes in eukaryotic organisms, such as transcription, duplication and DNA repair.[2] Monoubiquitination is an important histone PTM that controls many aspects of chromatin structure and activity.[3-7] In spite of the significant progress made in recent years, our understanding of the precise molecular mechanisms underlying the consequences of histone ubiquitination remains limited. The study of histone ubiquitination biology would benefit greatly from the availability of ample quantities of homogeneous ubiquitin-histones. The access to ub-histones is traditionally based on enzymatic methods or purification from cell source; however, these traditional approaches have some limitations, such as the requirement for a specific E3 ligase for each substrate, incomplete reaction or low yield, sample heterogeneity and possible co-existence of other naturally occurring modifications.[8] Chemical approaches provide an attractive alternative, and several strategies have been developed to conjugate ubiquitin to histones via non-native linkages, including an isopeptide bond with the ubiquitin C-terminal Gly76 mutated to Ala,[9] a disulfide bond,[10] or cross-linked mimics formed by 1,3-dichloroacetone.[11] While these non-native-linkage analogs are very useful,[9-12] they may not reflect all the genuine features of their native counterparts. To make the native ubiquitinated histones, Muir's group first reported the semisynthesis of H2B ubiquitinated at K120 and used the synthetic material to demonstrate the crosstalk between H2B K120 ubiquitination and H3 K79 methylation.[13] Brik's group reported the total synthesis of H2BK34ub through δ-thiolysine mediated chemical ligations.[14] Although this total synthesis work represents the latest advance in peptide chemistry, it is technically very demanding. Clearly, a more convenient approach would be one in which site-specific ubiquitination can be achieved directly on recombinant histones.




**Scheme 1.** Semisynthesis of ubiquitinated H2A at K119 position achieved by a genetically incorporated azidonorleucine (ANL) using methionine auxotrophic *E. coli* strain and subsequent $N^{\alpha}$-auxiliary-mediated native chemical ligation (NCL). Reagents and conditions: (i) Boc anhydride, DIEA, DMSO; (ii) 1 M TCEP in $H_2O$, DMSO; (iii) **9**, DIEA, DMSO; (iv) TFA/TIS/$H_2O$ (95/2.5/2.5); (v) 6 M Gdn.HCl, 0.2 M phosphate, 0.4 M $MeONH_2$, pH 4.0; (vi) Ub(1–75)-MES, 6 M Gdn.HCl, 0.2 M phosphate, 25 mM TCEP, 25 mM MPAA, pH 8.0; (vii) TFA/TIS/$H_2O$ (95/2.5/2.5).

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We recently developed a highly efficient and chemoselective method for native chemical ubiquitination of recombinant proteins.[15] Built on the work by Tirrel's group on the genetic incorporation of azidonorleucine (ANL) into proteins,[16] this method makes use of ANL as a lysine precursor to selectively install onto it a Gly residue bearing an acid-labile ligation auxiliary for introducing ubiquitin into the protein substrate.[15] The low abundance of methionine (Met) in histones makes them particularly amenable to azidonorleucine (ANL) replacement expression in Met-auxotrophic *E. coli* strains. Herein, as one demonstration of our strategy, we chose H2A, which has no methionine, as our target protein and Lys119 as the site of ubiquitination (Scheme 1). The large number of Lys residues (13 in total) in H2A makes it a perfect choice to test our semi-synthetic strategy for site-specific ubiquitination. The prevalent H2AK119ub is associated with a repressed chromatin state and is involved in Polycomb silencing.[5,17] We envisioned that the semisynthetic ubH2A with a native isopeptide linkage would help the study of deubiquitinases (DUBs) and crosstalk with other modifications.

To increase the incorporation efficiency of ANL, the gene of a triple mutant methionyl-tRNA synthetase L13N-Y260L-H301L (MetRS-NLL) previously reported by Tirell's group was generated due to its high activity for ANL.[16d] To site-specifically introduce ANL into *Xenopus laevis* H2A, the codon for K119 was mutated to that for Met. Because the second residue in H2A is a small amino acid serine, we predicted that the initiating Met substitute for ANL would be removed in situ after protein expression in *E. coli*.[18] Protein expression of the H2A-K119M gene was carried out under the co-expression of MetRS-NLL in *E. coli* grown in minimal media supplemented with 1 mM ANL. The yield of H2A **1** was about 12 mg/L after size-exclusion and C4 Preparative RP-HPLC purification. Electrospray ionization mass spectrometry (ESI-MS) analysis of the protein confirmed the quantitative incorporation of ANL into H2A and the removal of initiating ANL as predicted (Fig. 1B, trace a).

Next, a global protection strategy was employed to protect all the free amino groups in H2A **1** with Boc groups. There are 12 lysine residues and a free N-terminal amine in H2A **1**. Despite the high number of free amines, Boc protection was accomplshed efficiently in basic DMSO solution in 1 h at room temperature. ESI-MS result (Fig. 1B, trace b) showed that fifteen Boc groups were added onto H2A **1**. This indicated the full protection of all the free amino groups of H2A **1** and that the observed two additional Boc groups were due to protection of the two histidine residues (His31 and His82) in H2A. After Boc protection, the azide group of ANL at position 119 was reduced to the primary amine by tris(2-carboxyethyl)phosphine hydrochloride (TCEP) in DMSO for 3 h at room temperature. ESI-MS analysis showed that azide group was reduced quantitatively and that there were thirteen and fourteen Boc groups present on the reduced protein (Fig. 1B, trace c). The fewer number of Boc groups was due to the labile nature of Boc on the imidazole nitrogen of the two His residues. The installation of the ligation auxiliary onto the revealed ε-amino group of Lys119 in H2A was done using

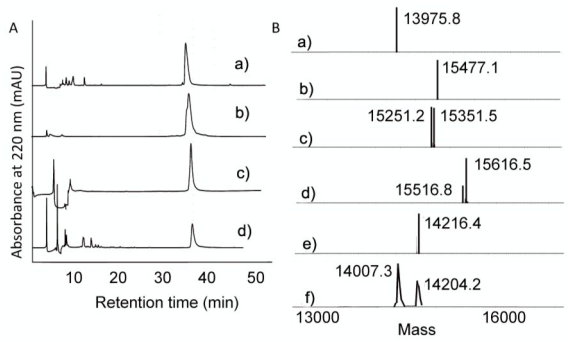


**Fig. 1** C8 analytical HPLC (A) and deconvoluted ESI-MS (B) of the key intermediates during the installation of auxiliary to the side chain of H2A K119. (A): (a) purified H2A **1**; (b) crude H2A **5**; (c) purified H2A **5**; (d) purified H2A **6**. Gradient: 0–80% buffer B (90% acetonitrile, 10% $H_2O$ containing 0.045% TFA) in buffer A ($H_2O$ containing 0.045% TFA) for 60 min. (B) (a–f): Deconvoluted ESI-MS of H2A **1**-**6**. Average mass of **1**, calculated 13976.02, observed 13975.8; average mass of **2**: Boc15, calculated 15477.76, found 15477.1; average mass of **3**: Boc13, calculated 15250.52, found 15251.2, Boc14, calculated 15350.64, found 15351.5; average mass of **4**: Boc13, calculated 15515.85, found 15516.8, Boc14, calculated 15615.97, found 15616.5; average mass of **5**: calculated 14215.36; found 14216.4; average mass of **6**: calculated 14203.35; found 14204.2. At the same time, 14007.3 species was also observed due to the removal of the auxiliary during ESI-MS detection.

reagent **9** (Scheme 1, step iii). The installation was completed within 2 h, as judged by ESI-MS analysis (Fig. 1B, trace d). Subsequently, all Boc groups were removed by treatment with TFA for 20 min at room temperature. The crude deprotected product was recovered by two times of ether precipitation and washing. C8 analytical HPLC and ESI-MS analysis of this sample indicated an excellent purity profile of the desired product H2A **5** (Fig. 1A, trace c and 1B, trace e). Detailed procedures for the preparation of H2A **5** from H2A **1** are given in Supplementary Information. Subsequent treatment of purified H2A **5** with 0.4 M methoxylamine in 6M Gdn.HCl (0.2 M

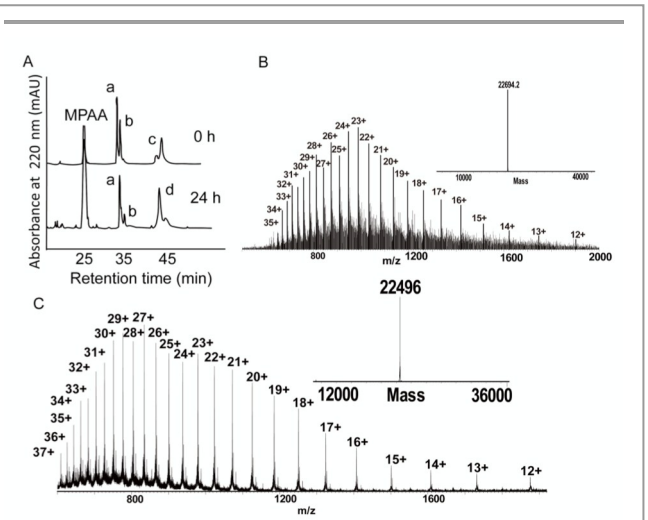


**Fig. 2** (A) C8 analytical HPLC monitored ligation reaction between H2A **6** and ub75-MES with MPAA as the thiol additive at 0 h and 24 h. Peak a: ub75-MES; peak b: ub75-MPAA; peak c: H2A **6**; peak d: ligation product H2A **7**; (B) The raw and deconvoluted ESI-MS of H2A **7**. Average mass calculated 22693.02; found 22694.2; (C) ESI-MS analysis of H2A **8**. Average mass calculated 22496.75, found 22496.0.

phosphate, pH 4) for 5 h at 37 ℃ led to quantitative deprotection of the thiazolidine on Lys119. The thiazolidine deprotection was confirmed by ESI-MS (Fig. 1B, trace f). The overall yield of HPLC-purified H2A **6** from H2A **1** was estimated to be 39.3%.

Next, we focused on the ubiquitin ligation reaction between the ubiquitin α-thioester and H2A **6**. The truncated *Homo sapiens* Ub75 α-thioester (containing residues 1-75) was prepared biosynthetically as an Ub75/intein/CBD (chitin-binding domain) fusion protein by on-column thiolysis using 2-mercaptoethanesulfonic acid (MESNA).[19] Under the previously described ligation conditions,[15] complete ligation of the two proteins was observed after 24 h, and ubH2A **7** was isolated in 70% yield and confirmed by ESI-MS (Fig. 2 A and B). To obtain ubH2A **8**, a cocktail of TFA/TIS/$H_2O$ (95/2.5/2.5) was used to remove the auxiliary for 20 min on ice. The purified final product ubH2A **8** had the desired molecular mass with excellent purity profile as confirmed by ESI-MS analysis (Fig. 2C). SDS-PAGE analysis of ubH2A **8** revealed a pure band at approximately 22 kDa, which was detectable by western blot analysis with anti-ubH2A Lys119 specific antibodies (Fig. 3A), further confirming the site of ubiquitination. Using this protocol, 3.3 mg of ubH2A was generated starting from 8.8 mg of the expressed recombinant protein H2A **1** in an overall yield of about 23%.

To demonstrate that the semisynthetic ubH2A possesses the characters of the natural counterpart purified from cells, we incubated the synthesized ubH2A with ubiquitin C-terminal carboxy hydrolase (UCH-L3) to see if it could be deubiquitinated. SDS-PAGE analysis showed that ubiquitin and H2A were released after UCH-L3 treatment for 30 min (Fig. 3B). This result indicated the presence of native isopeptide bond linkage in the synthesized ubH2A. Encouraged by this result, we continued to reconstruct nucleosome core particles (NCP) containing ubH2A and other *Xenopus laevis* core histones (H2B, H3 and H4) using the standard procedure.[20] Native PAGE analysis showed that ubiquitinated NCP has an upper shift on the gel compared with the unmodified NCP (Fig. 3C). Then we used ubiquitinated NCP as physiologically relevant substrate to test its behaviors towards two DUBs. Western blot results showed that ubiquitinated NCP was almost completely hydrolyzed by USP21 within 10 min. On the other hand, it could not be deubiquitinated by the recombinant BRCA1 associated protein 1 (BAP1) (Fig. 3D). Both USP21 and BAP1 are known H2A-specific DUBs in vitro and in vivo.[21] Our study shows that BAP1 alone does not have DUB activity towards ubiquitinated NCP in vitro. This observation is consistent with a previous report that BAP1 removed monoubiquitin from enzymatically generated ubH2A only in the presence of additional protein partners.[22] Besides BAP1 and USP21, some other DUBs including USP3 and USP16 are also known to perform the same function but are actively involved in different cellular events.[23] Hence, generating native ubiquitinated H2A can have important implications in biochemically characterizing these DUBs, and for identifying other ubH2A-interacting proteins or protein complexes. Clearly, this cannot be done with other synthetic ub-histone analogs linked through non-isopeptidic linkages.

In a recent study by Zhu's group using a 12-mer oligonucleosome array reconstituted from *Homo sapiens* histones and containing uniformly K119-ubiquitinated H2A (which was purified from mammalian cells), it was shown that H2AK119 ubiquitination negatively regulated H3K36 methylation levels through inhibiting the activities of several H3K36-specific methyltransferases.[24] However, the question remains as to whether the inhibition is caused by the ubiquitin present in the same or a neighboring nucleosome in folded oligonucleosome. This question can be easily addressed by performing the same experiments on ubiquitinated mononucleosomes. It is also interesting to know whether this negative crosstalk holds true in systems reconstituted from non-human histones such as the one from *Xenopus laevis*. So the NCP system we prepared provided a good opportunity to address these questions. Therefore, the enzymatic activity of NSD2 and SETD2 was tested on both unmodified and ubiquitinated NCP. NSD2 is the H3K36-specific methyltransferase (MTase) and it is mainly responsible for di-methylation of H3K36.[25] SETD2 mainly serves to tri-methylate H3K36.[26] Our results showed that detected levels of H3K36 di and tri-methylation were significantly decreased on ubiquitinated NCP as compared to the unmodified NCP (Fig. 4 A and B). To further verify that the decreased methylation level of H3K36 in ubiquitinated NCP was indeed caused by the ubiquitin, we decided to remove ubiquitin from ubH2A in NCP using USP21. After USP21 treatment, western blot analysis confirmed the near complete removal of ubiquitin from ubH2A (Fig. 4 C and D). Crosstalk assay showed again that the removal

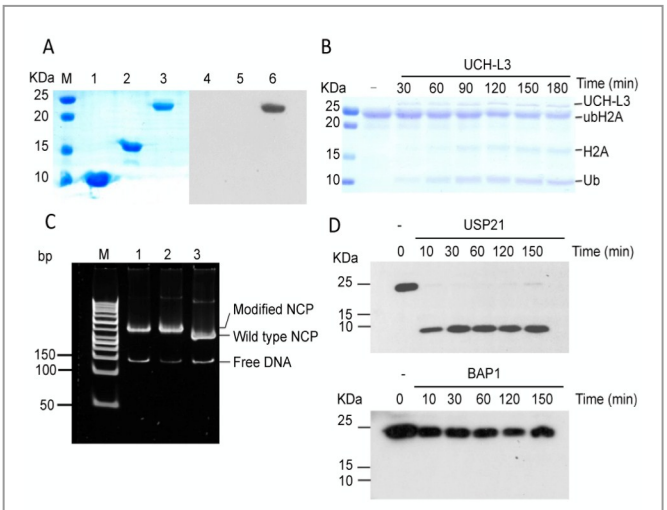


**Fig. 3** (A) SDS-PAGE and western blot analysis of ubiquitin, wild type H2A, and H2A 8. Lane 1 and 4 is ubiquitin; Lane 2 and 5 is wild type H2A; Lane 3 and 6 is ubH2A 8; (B) SDS-PAGE analysis of UCH-L3-mediated hydrolysis of ubH2A at 30, 60, 90, 120, 150 and 180 min and control was performed without UCH-L3; (C) Native PAGE analysis of wild type and ubiquitylated NCP. Lane 1 and 2 is ubiquitylated NCP; Lane 3 is wild type NCP; (D) Deubiquitylation of ubH2A by USP21 and BAP1 monitored by western blot using anti-ubiquitin antibody at 10, 30, 60, 120, 150 min and control was performed without USP21 or BAP1.

of ubiquitin by USP21 treatment restored the dimethylation and trimethylation levels of H3K36 as they were significantly higher than those without USP21 treatment (Fig. 4 C and D). For some unknown reason, a degradation band of methylated H3 was always detected under the test conditions. This phenomenon was also observed in the previous study by Zhu's group.[24]

Consistent with earlier findings by Zhu et al.,[24] our data further confirm the inhibitory role of H2A ubiquitination on H3K36 methylation by the specific MTases in mononucleosomes reconstituted from *Xenopus laevis* histones. This further explains the observation that ubH2A and H3K36 methylation rarely coexist in vivo.[24] The fact that the effect was observed in the NCP context also reveals the intra-nucleosomal nature of the cross-talk. It is unclear how H2AK119Ub exerts its inhibiting effect on H3K36 methylation by the MTases. With H2AK119 in relatively close proximity to H3K36 in NCP, ubiquitination of H2AK119 might simply present a steric block to the binding of MTases. Another possibility is that the presence of the H2AK119 ubiquitin might have caused a conformational change of the nucleosome, making the H3 site an unfit platform for MTase binding. Only future structural studies on such systems will be able to explain exactly how this crosstalk works. Overall, our crosstalk study provides another example that chemically synthesized ub-histones can help decipher mechanisms underlying the regulatory functions of histone ubiquitination.

In summary, our semi-synthetic ubiquitination method using a genetically encoded azidonorleucine was successfully used to synthesize biologically functional ubH2A. This method is generally useful regardless of the position of ubiquitination site in the histones. In principle, it should also be adaptable to synthesizing histones modified with other ubiquitin-like proteins such as SUMO and NEDD8.[27] We have also demonstrated the utility of the synthetic ubH2A in DUB assays and cross-talk studies with MTases. Considering the lack of structural information on ubiquitinated histones,[28] we anticipate that our method will help facilitate the challenging task of studying the effects of histone ubiquitination on chromatin structure by X-ray crystallography and cryo-electron microscopy. As the dysregulation of many of the enzymes that mediate the histone epigenetic reactions, such as ubiquitination/deubiquitination and methylation/demethylation, is implicated in human diseases, the availability of the chemically unambiguous ubiquitinated histones and NCPs will also help in the discovery of specific inhibitors against these enzymes for drug development,[29] in a way similar to what is done in targeting the more studied ubiquitin-proteasome protein degradation system for cancer therapy.[30] One limitation with our method is the requirement for global protection of the full-length protein before installing the ligation auxiliary onto the target protein, which may limit the synthesis to small-to-medium-sized proteins such as histones. It remains to be seen as to whether or not it can be used to ubiquitinate larger proteins, especially those rich in lysine residues.

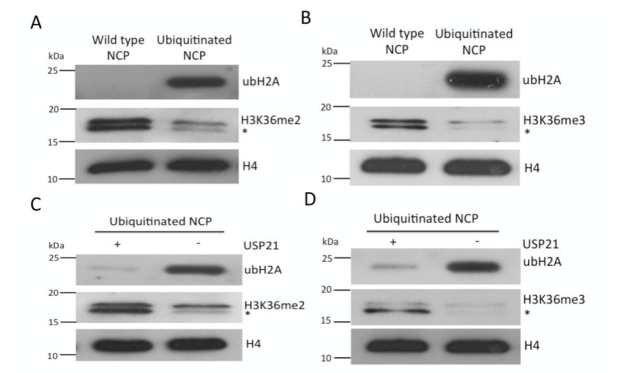


**Fig. 4** (A) H2A ubiquitination decreased the dimethylation level of H3K36 by inhibiting the activity of NSD2; (B) H2A ubiquitination decreased the trimethylation level of H3K36 by inhibiting the activity of SETD2; (C) Deubiquitination of ubH2A restored the dimethylation level of H3K36 mediated by NSD2; (D) Deubiquitination of ubH2A restored the trimethylation level of H3K36 mediated by SETD2. The asterisk indicates the detected degraded H3. H4 was used as the internal standard to compare the amount of wild type and ubiquitinated NCP.

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