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Chemical Synthesis of K34-Ubiquitylated H2B for Nucleosome Reconstitution and Single-Particle Cryo-Electron Microscopy Structural Analysis

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Post-translational modifications (e.g., ubiquitylation) of histones play important roles in dynamic regulation of chromatin. Histone ubiquitylation has been speculated to directly influence the structure and dynamics of nucleosomes. However, structural information for ubiquitylated nucleosomes is still lacking. Here we report an alternative strategy for total chemical synthesis of homogenous histone H2B-K34-ubiquitylation (H2B-K34Ub) by using acid-cleavable auxiliary-mediated ligation of peptide hydrazides for site-specific ubiquitylation. Synthetic H2B-K34Ub was efficiently incorporated into nucleosomes and further used for single-particle cryo-electron microscopy (cryo-EM) imaging. The cryo-EM structure of the nucleosome containing H2B-K34Ub suggests that two flexible ubiquitin domains protrude between the DNA chains of the nucleosomes. The DNA chains around the H2B-K34 sites shift and provide more space for ubiquitin to protrude. These analyses indicated local and slight structural influences on the nucleosome with ubiquitylation at the H2B-K34 site.

Histone ubiquitylation plays important roles in regulating many DNA-related cellular processes, such as DNA replication, DNA repair, and nucleosome reassembly. Among the multiple lysine sites on histone H2B, ubiquitylation at Lys120 (H2K120ub) has been studied extensively.[1] Recently, ubiquitylation at Lys34 of histone H2B (H2B-K34Ub)[2] gained much attention in facilitating relevant protein modification activities and regulating gene transcription, such as regulating Pol II processivity in a manner of feedback regulation through transcription elongation factors PAF1C and pTEFb.[2,3] H2B-K34Ub could directly stimulate the activity of histone methyltransferases (HMTases) involved in regulating histone H3 methylation.[2–3] In addition to providing a binding platform for related factors, post-translational modification of histones, especially in or near the structured histone fold (e.g., H2B-K34Ub), is considered to directly influence nucleosome structure and dynamics.[4] However, the precise regulation mechanism of histone ubiquitylation remains elusive, mainly due to difficulties in structural studies of nucleosomes with ubiquitin modifications.

Biochemical studies of ubiquitylated nucleosomes have been conducted by using chemically synthesized ubiquitylated histones. Muir and coworkers employed disulfide-linked ubiquitylated H2B to reveal that H2B-K120Ub could disrupt local and higher order chromatin compaction.[5] Through semi-synthetic H2B-K120Ub and H2A-K119Ub, they also showed that histone ubiquitylation could marginally alter the stability of nucleosomes.[6] Recently, several structures of ubiquitylated nucleosome and its complex were determined through X-ray crystallography or cryo-EM analysis.[7] The crystal structure of deubiquitinating enzyme bound to nucleosomes containing H2B-K120ub provided hints about the structural influence of K120-ubiquination on nucleosomes.[7a] The crystal structure of nucleosomes containing H2B-K120Ub and H4K31Ub revealed that the linked ubiquitin domains were flexible.[7b] Also, the cryo-EM structure of 53BP1 fragment bound to H4K20me2 and H2A-K15Ub-containing nucleosomes revealed the basis of 53BP1 recruitment to double-stranded DNA breaks sites.[7c] However, structural information for nucleosomes with ubiquitin modification on other lysine sites is still required, which would shed light on the molecular mechanism of histone ubiquitylation recognition and further cellular function regulations.

To avoid difficulties of conventional enzymatic methods, several different chemical synthesis methods,[8] including expressed protein ligation,[1b,9] total chemical synthesis,[10] and amber suppression mutagenesis,[11] have been used to produce homogeneous ubiquitin-modified histones with a native isopeptide linkage. By using these reported methods, the ubiquitin was eventually linked to histone proteins through reversible auxiliary-[12] or thiolysine[13]-mediated ligations.

Here, we developed an alternative method for total chemical synthesis of ubiquitylated histones involving trifluoroacetic acid (TFA)-labile 1-(2,4-dimethoxyphenyl)-2-mercaptoethyl aux-

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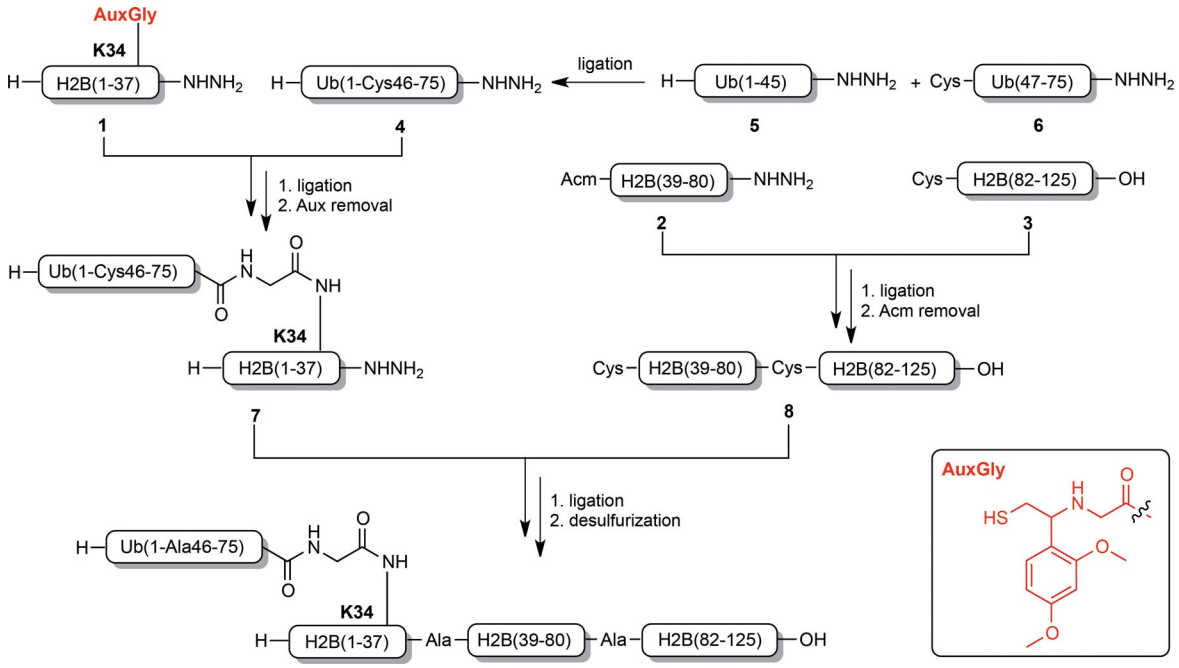

Scheme 1. Synthetic scheme for ubiquitylation of histone H2B at Lys34. Auxiliary-mediated ligation of peptide hydrazides was used to ligate peptide **1** to peptide **4**, forming intermediate **7** after auxiliary removal. Ligation between peptides **2** and **3**, followed by Acm removal, formed intermediate **8**. Ligation of intermediate **7** resulted in **8**, followed by free radical desulfurization to form full-length H2B-K34Ub.

iliary (Aux)[14] for site-specific ubiquitylation (Scheme 1). This alternative auxiliary was easily synthesized (two steps) and incorporated into peptides, and it exhibited high efficiency of ligation at Gly-Gly sites.[14] The synthetic H2B-K34Ub was reconstituted into the nucleosome and used for cryo-EM analysis.

H2B-K34Ub (Sequence shown in Figure S1 in the Supporting Information) was divided into five segments as shown in Scheme 1, namely H2B[Pro1-K34AuxGly-Tyr37]-$NHNH_2$ (**1**), H2B[Cys(Acm)38–Leu80]-$NHNH_2$ (**2**), H2B[Cys81–Lys125]-OH (**3**), Ub[Met1–Phe45]-$NHNH_2$ (**5**), Ub[Cys46–Gly75]-$NHNH_2$ (**6**). These segments were assembled through a convergent strategy, with Cys38 in segment **2** protected by an acetamidomethyl (Acm) group. To achieve ubiquitylation at Lys34, AuxGly was coupled to the side chain of Lys34 in segment **1** through 9-fluorenylmethoxycarbonyl (Fmoc) solid phase peptide synthesis (SPPS) (Figure S6). With all segments synthesized through Fmoc-SPPS, the protein synthesis was initiated through condensing segments **5** and **6**, producing the ubiquitin monomer of Ub[Met1–Cys46–Gly75]-$NHNH_2$ (**4**). Then, segment **4** was ligated to segment **1** by Aux-mediated ligation of peptide hydrazides (Figure S10). Subsequently, intermediate **7** was produced after treatment by TFA. At the same time, intermediate **8** was obtained by condensing segments **2** and **3**, followed by efficient removal of the Acm group. Then, ligation between segments **7** and **8** was implemented to produce the full-length peptide. The final target protein was obtained through a VA-044-initiated free radical desulfurization reaction.[16] The molecular weight and purity of the synthesized H2B-K34Ub was confirmed by ESI-MS, SDS-PAGE, and HPLC analysis (Figure 1 A). Following these procedures, 15–25 mg of H2B-K34Ub was obtained (total isolated yield ~11 %).

Next, the synthesized H2B-K34Ub was used to assemble histone octamers with recombinant histone H2A, H3, and H4

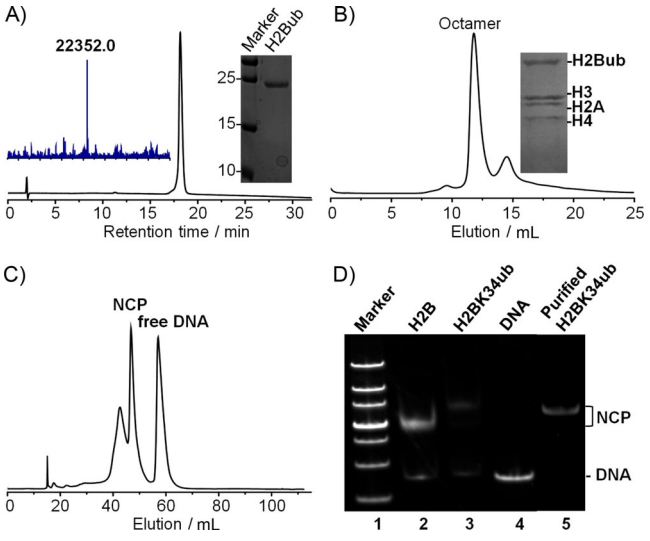

Figure 1. Reconstitution of mono-nucleosome with synthetic H2B-K34Ub. A) HPLC trace (214 nm), deconvoluted ESI-MS and SDS-PAGE analysis for synthetic H2B-K34Ub: $[M+H]^+$ observed = 22352.0 ± 1.3 Da; calcd. 22350.8 Da (average isotopes). B) Octamers containing H2B-K34Ub were purified by size-exclusion chromatography. Purified octamers were analyzed by using SDS-PAGE and stained with Coomassie Brilliant Blue. C) Mono-nucleosome purification with anion-exchange column (DEAE). Homogeneous nucleosome core particle (NCP) and free DNA were marked. D) Native gel analysis of mono-nucleosomes: marker (lane 1), NCP containing unmodified H2B (lane 2), unpurified NCP containing H2B-K34Ub (lane 3), free DNA (lane 4), purified NCP containing H2B-K34Ub (lane 5).

through gradient dialysis, followed by size-exclusion chromatography purification (Figure 1B).[17] Subsequently, the octamers were successfully reconstituted into nucleosomes with 147 base pairs of the 601 nucleosomal targeting sequence[18] by stepwise dialysis (Figure 1C and D).[17] The purified nucleosomes (lane 5 in Figure 1D) were immediately subjected to flash freezing, and single-particle cryo-EM pictures were recorded with a TITAN Krios instrument equipped with a Falcon-3 direct electron detector.

A total of 240456 cryo-EM particle images were selected for 2D image classification and 3D structure classification (Figure S13). Because each histone octamer could bind two H2B proteins, we had expected to observe two extra densities of ubiquitin domains in each particle, compared with that of the native nucleosome structure (PDB ID: 1KX5).[19] Surprisingly, among all of the 107000 particles, only 9324 particles were classified into a 3D structure containing two protruding domains (type 3), and 18502 particles contained a single protruding domain (type 2). The largest group, with 79550 particles, belonged to a reconstruction showing no obvious protruding domains (type 1; Figure S13). Fitting the crystal structure of the native nucleosome into the cryo-EM map indicated that the protruding domain was the attached ubiquitin domain in the nucleosomes containing H2B-K34Ub. We subsequently subjected these three groups of particles to structural refinement to obtain three structures (Figures 2A and S14). The refined density maps had a resolution of 7.6 Å (Figure 2A, type 1), 16.0 Å (Figure S14a, type 2), and 17.6 Å (Figure S14b, type 3), respectively, based on the gold-standard Fourier shell correlation criterion (Figure S13). The crystal structure of the unmodified nucleosome (1KX5)[19] fitted well into the three cryo-EM structures in a rigid body except that the histone tails were not observed in the cryo-EM maps, likely due to their flexibility in solution.

Unfortunately, in the refined type 1 structure, only smearing densities could be observed around the ubiquitylated sites of H2B. This implied that the linked ubiquitin was not observed in the cryo-EM structure. One explanation is that the ubiquitin might be cleaved prior to sample freezing for cryo-EM imaging. However, ESI-MS and SDS-PAGE analyses (Figure 1A) clearly demonstrated that the synthetic H2B-K34Ub retained high integrity, with correct molecular weight and high purity. Meanwhile, the synthetic H2B-K34Ub was still stable in the reconstitution of octamer and nucleosome in vitro, and no contaminated H2B could be found in the SDS-PAGE analysis (Figure 1B and D). Therefore, the absence of ubiquitin densities in type 1 particles could only be attributed to the flexibility of ubiquitin domains, in contrast to the high rigidity of octameric histones in nucleosomes. The conformation flexibility led to the absence of ubiquitin densities during structure averaging, consequently resulting in more particles without protruding domains (79.5 K/117 K) but relative high resolution (7.6 Å). In the other two refined structures, one or two protruding domains were observed that were smaller than the docked crystal structure of ubiquitin (PDB ID: 1UBQ)[20] and much weaker than the density assigned to the nucleosome with low resolution (17.6 and 16 Å, Figure S14), strongly indicating the high flexibility of the extra domain. Despite the low resolution of the nucleosomes

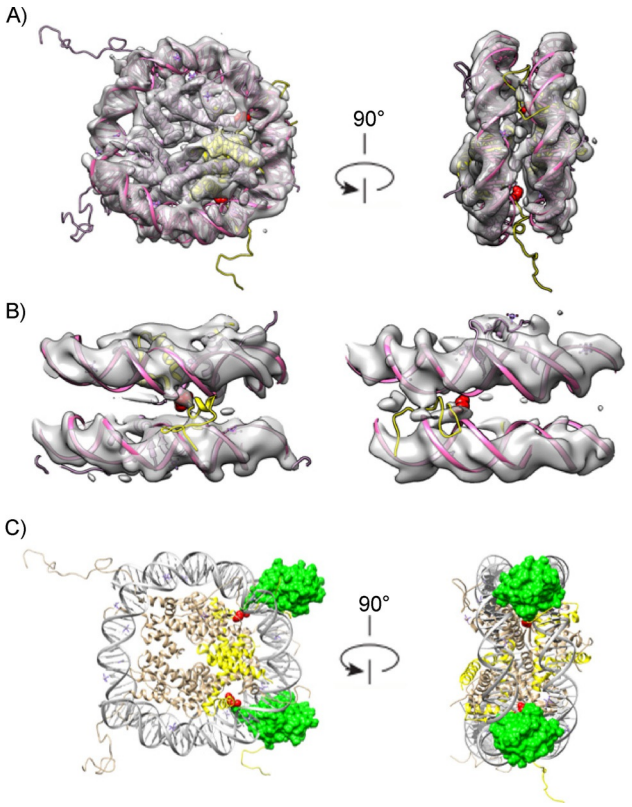

Figure 2. CryoEM structure (type 1) of the nucleosomes containing chemically synthesized H2B-K34Ub. A) The type 1 structure with no observable ubiquitin domain. The K34 sites in H2B proteins (marked yellow) are shown as red dots. B) DNA densities in the structure deviated from those in the nucleosome crystal structure. The cryo-EM densities are shown as semi-transparent surfaces with the rigid-body docked crystal structure of native nucleosome (PDB ID: 1KX5)[19] as ribbons. C) The structural model of nucleosome containing synthetic H2B-K34Ub. Ubiquitin domains (green, PDB ID: 1UBQ)[20] with high flexibility were hypothesized to protrude between two DNA chains and exerted limited influences over the main structure of nucleosome.

with protruding extra domains, it could still be hypothesized that the two ubiquitin domains were protruding between two DNA chains. This orientation conformed to the location of the H2B-K34 site that was located just beneath the DNA chain surrounding the histone octamers (Figure 2). The high flexibility of ubiquitin domains was also similar to that in the recent complex structure of the deubiquitinating module bound to ubiquitylated nucleosomes[7a] and structures of the nucleosome containing H2B-K120Ub or H4-K31Ub.[7b] Moreover, the structure revealed that the two ubiquitin domains were protruding between the two gyres of nucleosomal duplex DNA chains on the radial surface, which was different from reported results at other lysine sites.[7]

In general, the refined cryo-EM structure fit well with the native nucleosome structure (Figure 2A). The resolution of 7.6 Å of this cryo-EM map allowed us to assign some secondary structural elements in the cryo-EM densities by comparing with the crystal structure of the nucleosome (1KX5).[19] For example, the cryo-EM density of the long α-helix (Ile54–Asn84 segment) in the lower H2B matched well with the segment in

the crystal structure (Figure S15a), whereas the cryo-EM density of the Tyr37–His49 α-helix was slightly blurry (Figure S15b); this indicates that there are some conformational changes from the N terminus to His49. Moreover, with regard to the upper H2B in the nucleosome, the density maps of the long α-helix (Ile54–Asn84, Figure S15c) and the short α-helix (Tyr37–His49, Figure S15d) both fit well with the crystal structure, indicating a stable backbone conformation of the upper H2B after ubiquitylation. As shown in Figure 2B, it could be observed that the distances between the DNA chains around the potential protrusion ubiquitin neck in the type 1 structure was longer than that in the unmodified nucleosome. At the lower ubiquitin location, the two sides of DNA chains were deformed and expanded by ~3 Å (upward) and ~2 Å (downward) from its original location respectively, versus the nucleosome crystal structure (Figure 2B). The deformed DNA chains provided ~16% more space, which should be sufficient for the modified ubiquitin domain to pass through. On the upper protrusion side, both sides of the DNA surfaces also expanded by ~3 Å (Figure 2B). The total ~6 Å displacement could provide ~20% more space for ubiquitin to protrude.

The cryo-EM structure indicated that H2B-K34 ubiquitylation resulted in the deformation and expansion of the two DNA chains. We then performed a thermal stability assay[7b,21] to measure the stability of nucleosome containing H2B-K34Ub. The denaturation curves of unmodified and ubiquitylated nucleosomes (Figure 3) were obtained by monitoring the fluorescence signal of SYPRO Orange bound to thermally denatured histones.[7b,21] The curve of unmodified nucleosome (Figure 3A)

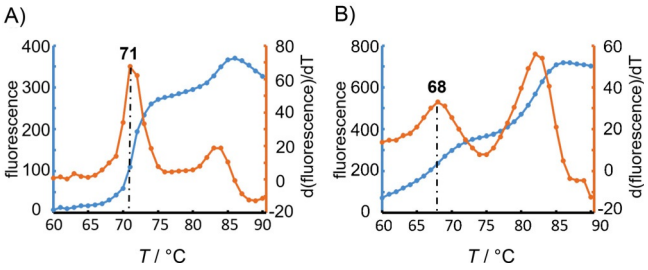

Figure 3. Thermal stability curves of the A) unmodified and B) H2B-K34Ub nucleosomes. Blue curves indicate the normalized fluorescence intensities against temperature. The standard deviation curves are plotted in orange. The temperatures corresponded to the dissociation of H2A-H2B from nucleosomes and are marked in the figures.

was consistent with the reported results, containing two peaks (71 and 83 °C) that corresponded to the release of H2A-H2B and H3-H4 from nucleosome, respectively.[7b,21] However, the first peak of nucleosome containing H2B-K34Ub was shifted towards a lower temperature (68 °C), whereas the second peak was not obviously shifted (Figure 3B). These results indicated that H2B-K34 ubiquitylation might destabilize the association of H2A-H2B with nucleosome. These results conformed to the cryo-EM structure, in which H2B-K34 ubiquitylation mainly changed the conformation of DNA chains surrounding the interface between DNA and H2A-H2B dimers (Figure 2A and B), although it did not affect the octameric structure (Figure S15).

Finally, we presented a structural model of reconstituted nucleosome containing synthetic H2B-K34Ub (Figure 2C). After ubiquitylation, the distance between the two DNA chains around the H2B-K34 sites might increase and provide more space for the modified ubiquitin domain to pass through. Although some local conformation changes occurred, the ubiquitylation exerted limited influences over the main structure of nucleosome that were consistent with the recent observations of slight intrinsic stability alternation of ubiquitylation at histone proteins (e.g., H2A-K119Ub, H4-K31Ub, and H2B-K120Ub).[6,7b] The modified ubiquitin domain could be very flexible in solution, and this feature could lead to high accessibilities for different ubiquitin recognition proteins. For example, the H2B-K34Ub ubiquitin recognition functions of different folded HMTases could stimulate methylations at two lysine sites (H3-K4 and H3-K79, Figure S16) over long distances in nucleosomes.

In summary, homogenous histone H2B-K34Ub was prepared in multi-milligram-scale by total chemical synthesis. In this strategy, site-specific ubiquitylation was achieved through TFA-labile auxiliary-mediated ligation of peptide hydrazides. Our cryo-EM structures of the reconstituted nucleosomes containing H2B-K34Ub indicated that two flexible ubiquitin domains protruded between nucleosome-wrapping DNA chains, and H2B-K34 ubiquitylation could lead to some nucleosome conformation changes and slightly affect the nucleosome structure. In particular, this study demonstrated an alternative strategy to prepare samples of nucleosomes with ubiquitin modifications, which provides a basis for further investigations of nucleosome conformation changes and related gene expression regulation mechanisms with ubiquitin modifications on various lysine sites of different histone proteins.

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