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Effects of histone H2B ubiquitylation on the nucleosome structure and dynamics

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ABSTRACT

DNA in nucleosomes has restricted nucleosome dynamics and is refractory to DNA-templated processes. Histone post-translational modifications play important roles in regulating DNA accessibility in nucleosomes. Whereas most histone modifications function either by mitigating the electrostatic shielding of histone tails or by recruiting 'reader' proteins, we show that ubiquitylation of H2B K34, which is located in a tight space protected by two coils of DNA superhelix, is able to directly influence the canonical nucleosome conformation via steric hindrances by ubiquitin groups. H2B K34 ubiquitylation significantly enhances nucleosome dynamics and promotes generation of hexasomes both with symmetrically or asymmetrically modified nucleosomes. Our results indicate a direct mechanism by which a histone modification regulates the chromatin structural states.

INTRODUCTION

Nucleosome is the basic organizing unit of chromatin in eukaryotic cells (1,2). Compacted chromatin structures repress vital biological processes including transcription and DNA replication. Several mechanisms including histone post-translational modifications (PTMs) (3,4) and adenosine triphosphate (ATP)-dependent chromatin remodeling activities are able to modulate chromatin accessibility and functions (5–8). For example, transient and orchestrated displacement and re-association of the H2A/H2B dimer by histone chaperones and ATP-dependent remodeling activities facilitate progression of RNA polymerase II through chromatin and play critical roles in transcription activation (9–13). Alterations in nucleosome flexibility and/or dynamics (such as nucleosome 'breathing' or partial unwrapping and rewrapping of nucleosomal DNA (13,14)) also contribute to transcription regulation by promoting transient DNA exposure and accessibility of transcription factors.

Most histone PTMs on flexible histone tails have minimal interactions with the nucleosome core in canonic nucleosome structure (1,2). They function primarily by recruiting downstream regulatory proteins (15–17), mitigating electrostatic shielding of positively charged histone tails (18,19), or impeding inter-nucleosome interactions that are necessary for chromatin compaction (e.g. acetylation of histone H4 lysine 16) (20). In contrast, histone PTMs within the nucleosome entry-exit and dyad regions have more pronounced effect on intra-nucleosome dynamics (15–17,21). Recent studies show that histone acetylation at nucleosome entry-exit by Piccolo (22) or p300 (23) promote spontaneous nucleosome unwrapping by weakening DNA–histone interactions. Furthermore, acetylation of H3K56, H3K115 or H3K122 near dyad axis increases both nucleosome 'breathing' and mechanical unwrapping without affecting overall nucleosome assembly or stability (24–27). Histone acetylation also lead to 2- to 3-fold increase of nucleosome eviction in response to stretching force in magnetic tweezer force-extension measurements (27). In addition to histone acetylation, H3 phosphorylation at Y41 or T45 near nucleosome entry-exit point increases transient unwrapping of nucleosome at a rate comparable to H3K56ac (24). Despite these studies, it remains unclear whether histone PTMs, especially those that do not alter charges (e.g. methylation) or introduce bulky moiety (e.g. ubiquitylation), are able to induce changes in nucleosome stability or assembly, and if so, what are the functional implications.

Histone ubiquitylation is one of the key epigenetic marks that are involved in vital biological processes (28,29). It serves as a regulatory signal that promotes or represses other histone modifications (30–34). It is also directly involved in disrupting high-order chromatin structures (35,36) and modulating chromatin functions in conjunction with other chromatin factors (37–41). H2B lysine 34 is one of histone residues that is ubiquitylated *in vivo* (42). We previously show that this modification is catalyzed by the male specific lethal (MSL) complex (43–45). Ubiquitylation of

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H2B K34 (H2BK34ub), just like H2B K120ub (K123 for budding yeast) by the RNF20/40 complex, plays important roles in transcription elongation (28,45–47). We show that the MSL complex coordinates with the PAF1 and pTEFb complexes to promote Pol II processivity (45). It also directly and indirectly regulates transcription-associated H3 K4/79me and H2BK120ub (44,45). Recent cryo-EM structure shows that H2B K34ub alters DNA–histone interactions within nucleosomes to accommodate two bulky ubiquitin moieties, which protrude out of nucleosome core particle (48). This modification modestly affects the melting temperature of nucleosomes, but not tetrasomes, in a thermal shift assay (48). This raises a question of whether and how the 76 amino-acid ubiquitin moiety of H2B K34ub affects nucleosome assembly, stability and dynamics and if this function is subject to further regulation.

To examine the function of H2B K34ub on nucleosome assembly and stability as well as the direct effects of H2B ubiquitylation (K120 or K34) on nucleosome dynamics, we establish a defined and traceable *in vitro* system with homogenous preparations of chemically-modified recombinant nucleosomes. We show that ubiquitylation of histone H2B K34, and to a lesser extent H2B K120, significantly enhances nucleosome dynamics and promote nucleosome conversion to hexasomes.

MATERIALS AND METHODS

DNA constructs, protein purification

Unmodified histone octamers were prepared as previously described (49). Histones H2B K34ub were synthesized as previously described (48,95,96). H2B K120ub histone octamers were prepared as previously described (30). The 147 bp 601 DNA was prepared from EcoRV digestion of plasmid containing 12 tandem repeats of 601 DNA (49). The 177 bp 601 was prepared *Sca*I digestion of the p601-177 × 12 plasmid containing 12 copies of 177 bp 601 DNA (50). The 146 bp 601 DNA was prepared from EcoRV digestion of plasmid containing a tandem repeats of 146 bp 5S DNA (51). Fragments were purified by polyethylene glycol (PEG) fractionation (52). Nicked p601-177 × 12 plasmid was prepared by digestion of supercoiled p601-177 × 12 with Nt.*Bst*NBI (NEB). Positively supercoiled p601-177 × 12 was prepared by ligation of nicked plasmid in the presence of 50–100 μg/ml netropsin for 24 h at 18°C (53). The resulting supercoiling was verified by in 1.2% agarose polyacrylamide gel electrophoresis (PAGE) containing 10 μg/ml chloroquine in gel and running buffer (53). The *Xenopus laevis* histone H1° cloned in pET3d (54) was expresses as described (54), and purified sequentially over Sephadex CM-50 (Sigma) and Sepharose-SP (Amersham) columns: elution with 0.4–1.0 M NaCl in buffer A (20 mM Tris–HCl, pH 8.0, 1 mM dithiothreitol (DTT), 1 mM phenylmethane sulfonyl fluoride (PMSF)) using 100 and 50 mM NaCl steps, respectively.

Nucleosome assembly and analysis

About 0.25–1 μg/μl nucleosomes were reconstituted by serial dilutions from 2M NaCl to final concentration of 100–200 mM NaCl (55). The assembly was analyzed on pre-electrophoresed 5.5% native polyacrylamide gel with subsequent SYBR Gold staining. Polynucleosomes were analyzed essentially the same except that the samples were digested with Thermo Scientific ScaI for 15–30 min at 26°C before loading. The unmodified hexasomes were obtained by diluting histone/DNA mixture from 2M NaCl to 1M NaCl, followed by directly resolving on native PAGE. This condition impedes sub-assembly of histone H2A-H2B dimers, which bind to the H3-H4 tetramer–DNA complex at 0.6–0.8 M NaCl (56).

To analyze histone content, the modified nucleosomes were resolved in native PAGE. Positions of nucleosome were identified by staining a narrow slice of the gel with ethidium bromide. Gel segments of interest were excised from unstained portion of gel, homogenized in 1.5 volumes of 10 mM Tris–HCl, pH 8.0, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.01% NP-40, 1 mM PMSF, 0–100 mM NaCl. After overnight incubation at 4°C, the supernatant was precipitated by trichloroacetic acid and resolved in 15% sodium dodecyl sulphate-PAGE (SDS-PAGE) with subsequent SYPRO Orange staining.

Competition assays

Reactions (typically 5 μl) contained assembled mononucleosomes (25–40 ng DNA) in nucleosome dilution buffer (10 mM Tris–HCl, pH 8.0, 1 mM EDTA, 1 mM DTT, 100 μg/ml bovine serum albumin, 0.01% NP-40, 1 mM PMSF, 100–200 mM NaCl) and recombinant mouse NAP1 (57) at molar excess over histone octamers or purified plasmid DNA at weight excess over histone octamers. After incubation for 2–3 h at 26 or 37°C, samples were mixed with one-fifth volume of 60% sucrose/0.005% xylene cyanole in 1× TE (pH 7.6) and resolved in 5.5% native polyacrylamide gel as above.

Micrococcal nuclease digestion

Assembled nucleosome (∼30 ng DNA) were digested with increasing amounts (typically 0.3/1/3/10/30/100 Kunitz units) of MNase (NEB) for 20 min at 26 or 37°C. DNA was purified and resolved on 6.5% polyacrylamide gel (37.5:1) in 1× TAE buffer (40 mM Tris-OH, 20.6 mM acetic acid, 5 mM sodium acetate, 2 mM EDTA) and stained with SYBR Gold.

Image quantification

Gel image was quantified by ImageJ 1.42 software. Densitometry tracing was done using ImageJ option 'Analyze Profile'. Quantification was performed using ImageJ option 'Analyze - Measure'. Background was reduced with ImageJ option 'Process -Subtract Background' at default setting.

Rigor and robustness statement

We have used good laboratory practices to ensure rigor and robustness. All experiments were performed at least three times to verify the conclusions and representative results were shown in figures.

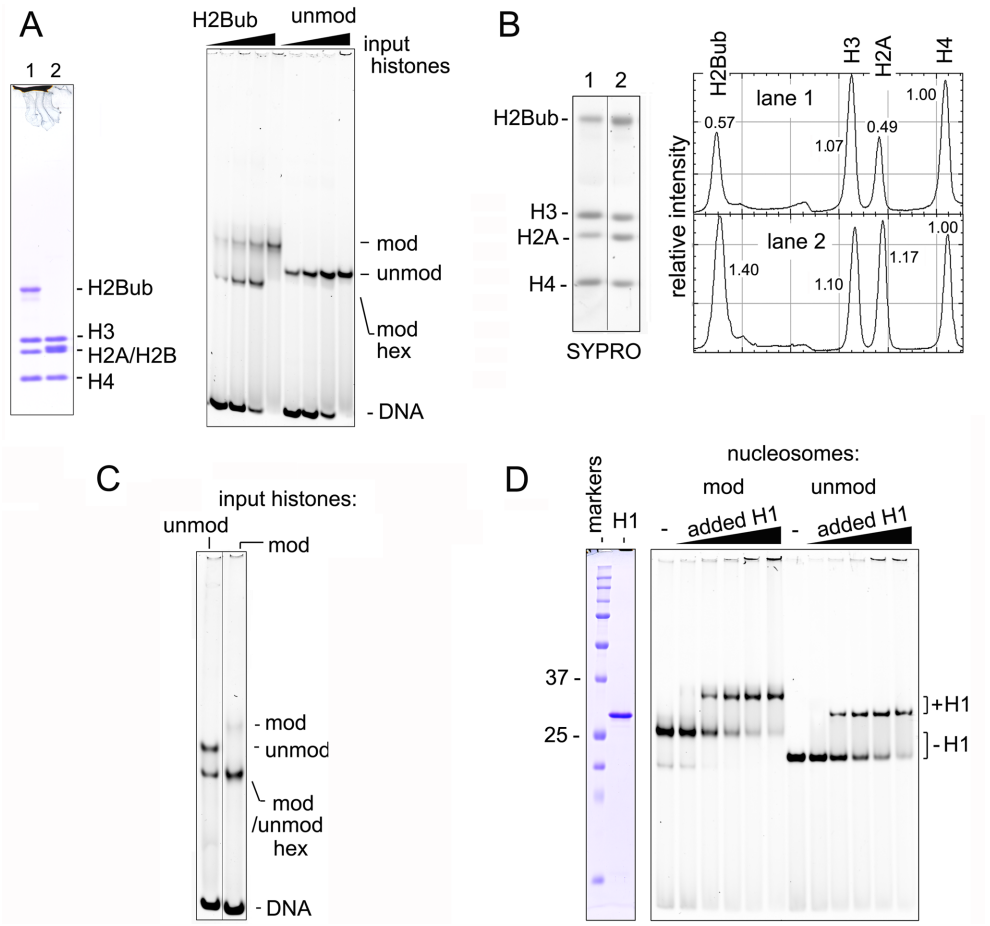

Figure 1. Assembly of H2B K34ub and unmodified nucleosomes. (**A**) Left panel: SDS-PAGE of H2B K34ub- (lane 1) and unmodified (lane 2) histone octamers. Right panel: native PAGE of modified nucleosomes assembled on 147 bp 601 DNA using increasing amounts histones. (**B**) SDS-PAGE of faster- (lane 1) and slower- (lane 2) migrating H2B K34ub assembly products extracted from the native gel in A. The densitometric tracing of the gel lanes is shown on the right. Quantification, by ImageJ, is normalized to that of the histone H4, which is arbitrarily set as 1. (**C**) Native PAGE for samples prepared by serial dilution of 2M NaCl mixtures of 147 bp 601 DNA and modified or unmodified histones to 1 M NaCl. (**D**) Left panel: SDS-PAGE of recombinant linker histone $H1^0$. Right panel: assembly of H2B K34ub modified and unmodified nucleosomes with increasing concentration of recombinant histone $H1^0$.

RESULTS

Assembly of H2B K34ub nucleosomes

To study the effects of histone H2B K34ub on nucleosome assembly and stability, we reconstituted nucleosomes using recombinant histone octamers with or without H2B K34ub as well as 147 bp DNA containing 601 nucleosome positioning sequence as previously described (55,58). H2B K34ub, prepared by peptide ligation (48), was integrated into histone octamer in stoichiometry (Figure 1A, left panel). Interestingly, while native histone octamers were efficiently reconstituted into nucleosomes, H2B K34ub-containing histone octamers were assembled into both nucleosomes and hexasomes (Figure 1A, right panel). Proteins extracted from native PAGE showed 50% stoichiometry of H2A/H2Bub dimer in hexasomes as compared to normal stoichiometry of the H2B K34ub-nucleosomes (Figure 1B). Notably, while H2B K34ub nucleosomes migrated significantly slower than the unmodified controls, mobility of H2Bub- or unmodified hexasomes was similar in the native gel (Figure 1C), suggesting that H2B K34ub probably fit compactly in the hexasome structure (between the DNA gyres) without significantly increase the Stokes radius. Furthermore, H2B K34ub did not abolish incorporation of linker histone $H1^0$ (Figure 1D) (59). These results indicate that while H2B K34ub can be stably incorporated into nucleosomes, cooperative assembly of H2A/H2B dimers is impaired.

Stability and dynamics of H2B K34ub nucleosomes

To quantify the effects of H2B K34ub on nucleosome assembly, we premixed modified and unmodified histone octamers at different ratios and assembled nucleosomes using 147 bp 601 DNA by serial salt dilution (Figure 2A and Table 1). Notably, both symmetrically and asymmetrically modified nucleosomes were assembled with much lower efficiency than what was expected from the molar ratio of modified versus unmodified histone octamers (Table 1). For example, when modified versus unmodified histone octamers was at 1:1 molar ratio, only 10% symmetrically modified nucleosomes were assembled, which was much less than the ex-

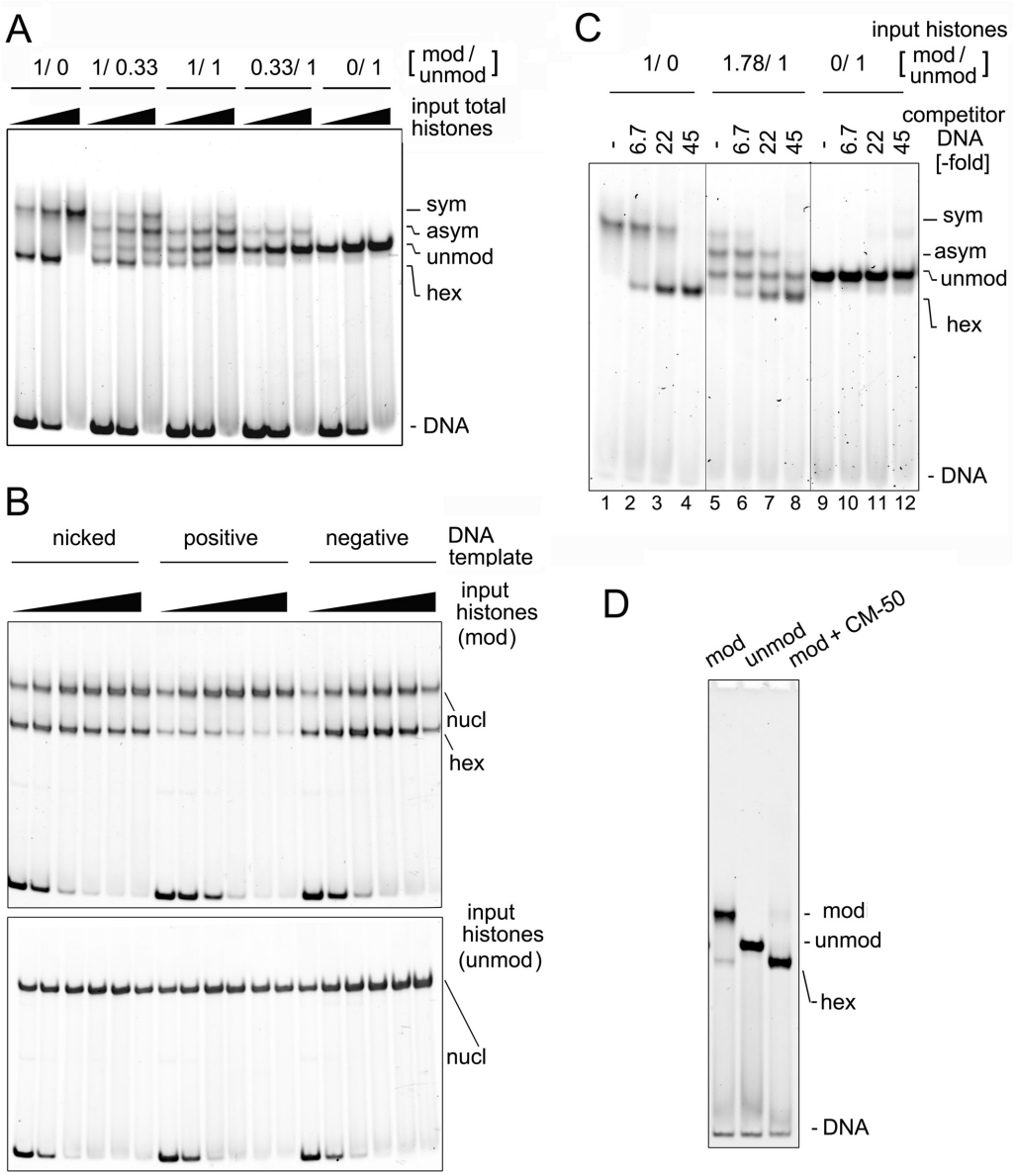

Figure 2. Stability of H2B K34ub nucleosomes. (**A**) Native PAGE for nucleosome assembly with increasing amounts of unmodified and H2B K34ub histone octamers and their mixture at ratios indicated on top. Quantification was performed by Image J and relative abundance of each species was summarized in Table 1. The representative result from three repeats was presented. (**B**) H2B K34ub (top panel) or Unmodified (bottom panel) nucleosomes were assembled on nicked, positively or negatively supercoiled plasmids containing 12 copies of 177 bp 601 DNA. After assembly 601 nucleosomes were released with ScaI and resolved on native PAGE. (**C**) The nucleosome assembly containing histones of indicated ratios (top) were incubated with competitor DNA for 2 h at 26°C in a buffer containing 200 mm NaCl. (**D**) Nucleosomes assembled on 147 bp 601 DNA (as indicated on top) were incubated with or without CM-50 Sephadex.

pected 25%. In contrast, over 50% unmodified nucleosomes was found in the assembly, more than the expected 25% (Table 1). We conclude that incorporation of H2B K34ub, symmetrically or asymmetrically, is less favored as compared to unmodified nucleosomes.

We next tested how DNA topology may affect stability of H2B K34ub nucleosomes. To this end, we used a plasmid DNA template with 12 copies of 177 bp 601 DNA (50) that were nicked or contained either negative supercoils or positive supercoils as previously described (53). Polynucleosomes were assembled by serial dilutions and then individual nucleosomes were released with ScaI and resolved on the native gel. As shown in Figure 2B, hexasomes formation was found in assembly of polynucleosomes containing H2B K34ub. Interestingly, compared to the nicked template, hexasome formation was affected by DNA topology. The positively supercoiled DNA, which tends to destabilize nucleosome structure (6,60,61), probably facilitates assembly of H2B K34ub nucleosomes. Whereas negatively supercoiled DNA, which usually promotes nucleosome compaction, enhances H2B K34ub induced nucleosome destabilization.

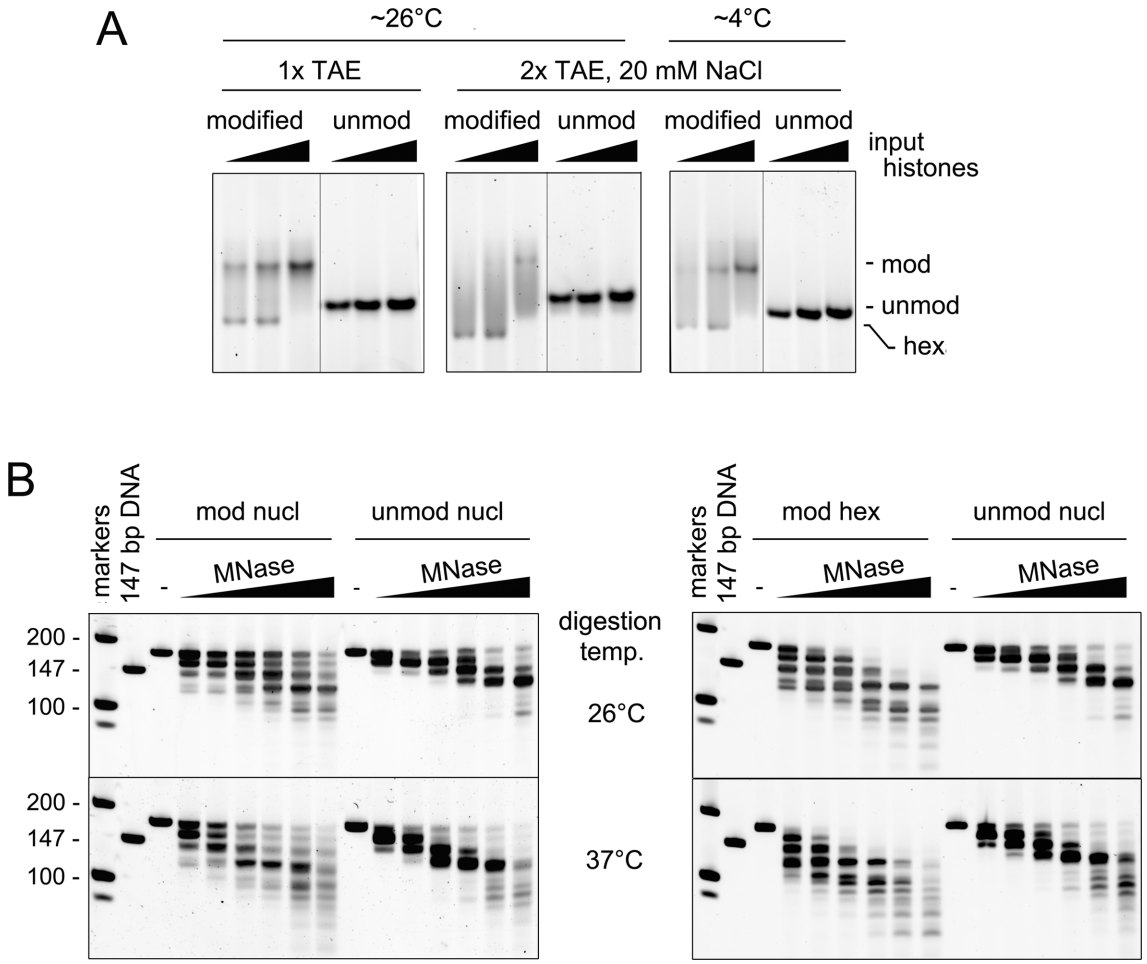

Figure 3. Dynamics of H2B K34ub nucleosomes. (**A**) Nucleosomes assembled on 147 bp 601 DNA were resolved in native PAGE under ionic and temperature conditions indicated on top. For the middle panel, although electrophoresis was performed at ∼26°C, the temperature at the glass surface was ∼35–37°C due to higher conductivity of the buffer. All gels were pre-electrophoresed in the relevant buffer. (**B**) Unmodified and H2B K34ub nucleosomes /hexasomes assembled on 177 bp 601 were digested with MNase at 26°C or 37°C and DNA was resolved in 6.5% PAGE and stained with SYBR Gold.

To test the function of H2B K34ub on stability of the H2A/H2B dimer within the nucleosome, we incubated the assembled symmetric-, asymmetric-H2BK34ub or unmodified nucleosomes with competitor plasmid DNA (Figure 2C). In the presence of competitor DNA, symmetrically modified nucleosomes were unstable and one H2A/H2B dimer was easily dissociated, resulting in a stable hexasome particle (Figure 2C, left panel). The asymmetrically modified nucleosomes were also less stable and converted to hexasomes in the presence of increasing amount of competitor DNA (Figure 2C, middle panel). As the control, unmodified nucleosomes were stable under the same conditions (Figure 2C, right panel). Consistently, H2B K34ub nucleosomes were converted to hexasomes by mixing with weak anion-exchanger Sephadex CM-50 (Figure 2D).

In addition to promote dissociation of one H2A/H2B dimer in the presence of DNA stress or competitor DNA, H2B K34ub was able to increase nucleosome dynamics. H2B K34ub-modified nucleosomes produced a diffused electrophoretic pattern when resolved under increasing ionic conditions and temperatures (Figure 3A). By lowering the gel running temperature, defined migration patterns of H2Bub nucleosomes was largely restored (Figure 3A). Furthermore, H2B K34ub nucleosomes exhibited 'expanded' MNase digestion profiles as compared to unmodified nucleosomes and this effect was more pronounced when MNase digestion was performed at 37°C (Figure 3B, left panel). These results probably indicate larger ensemble of structural conformation for H2B K34ub nucleosomes as a result of higher rate of transient unwrapping or 'breathing' of nucleosomes (13). Notably, the MNase protection pattern of H2B K34ub modified nucleosomes was not a simple result of dimer eviction since the modified hexasomes had different MNase digestion patterns (Figure 3B, right panel).

Histone dimer eviction in H2B K34ub nucleosomes by NAP1 protein

Histone chaperone NAP1 plays an important role in histone dimer exchange and displacement *in vivo* (62,63). To examine the ability of NAP1 to evict H2A/H2B K34ub dimer, we incubated 177 or 147 bp 601 nucleosomes with recombinant mouse NAP1 and the reaction products were resolved in native PAGE (Figure 4A). Consistent with higher intrin-

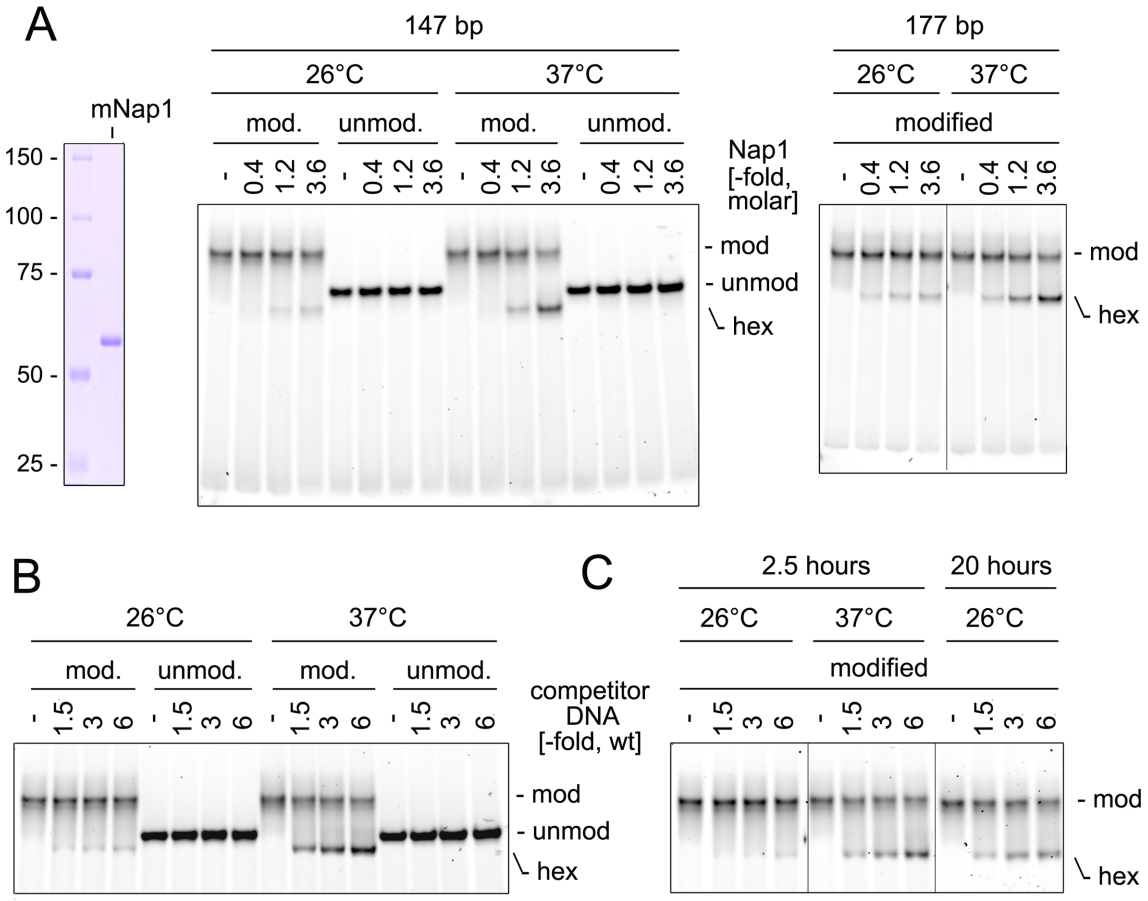

Figure 4. Eviction of histone dimer in H2B K34ub nucleosome by histone dimer acceptors. (**A**) Left panel: SDS-PAGE of NAP1. Middle, right panels: unmodified and H2B K34ub nucleosomes assembled on a 147 or 177 bp 601 DNA were post-assembly incubated with NAP1 for 2.5 h at 26 or 37°C in a buffer containing 100 mM NaCl. (**B**) Nucleosomes assembled on 147 bp 601 DNA were post-assembly incubated with competitor DNA for 2.5 h at 26/37°C in a buffer containing at 100 mM NaCl. (**C**) H2B K34ub nucleosomes assembled on 147 bp 601 DNA were post-assembly incubated with competitor DNA at 26/37°C for 2.5 h or at 26°C for 20 h (the '20 h' and '2.5 h' samples were resolved in different gels).

sic dynamics of H2B K34ub nucleosomes (Figure 3), NAP1 significantly enhanced H2B K34ub hexasome formation as compared to the unmodified nucleosomes (Figure 4A). NAP1-dependent dimer eviction was higher at 37°C (Figure 4A). Increased dimer eviction was also found by adding competitor DNA at 37°C (Figure 4B). Incubation of H2B K34ub nucleosomes with DNA at 26°C for 20 h led to similar level of dimer eviction as that of incubation at 37°C for 2.5 h (Figure 4C). Taken together, these results suggest that H2B K34ub increase nucleosome dynamics by promoting dimer transfer to NAP1 or competitor DNA and this process is kinetically controlled.

Stability of H2B K34- versus K120-ubiquitylated histone dimers in 601 and 5S nucleosomes

Position of H2B K34ub in nucleosomes is distinct from that of well characterized H2BK120ub. Previous study showed that H2B K120ub had minimal effects on stability of 601 nucleosome *in vitro* (64,65). However, when we compared H2B K34- versus K120-ubiquitylation on nucleosome assembly, there was increased hexasome formation in the H2B K120ub nucleosome assembly, albeit much less pronounced than that of H2B K34ub nucleosome (Figure 5A). Interestingly, H2BK120ub hexasomes migrated slower than H2B K34ub hexasomes, which might be due to conformational difference of the hexasome structures with ubiquitin moiety at different location. Incubation of the assembled nucleosomes with competitor DNA at different ionic and temperature conditions or with NAP1 showed that H2B K120ub only modestly promoted eviction of H2A-H2B dimers (Figure 5B and C), which was consistent with previous studies (64,65). To examine the effects of H2B K34ub versus K120ub on nucleosome dynamics using a more physiological DNA template, we assembled nucleosomes using the 147 bp positioning sequence from *Lytechinus* variegatus 5S rRNA gene (66). Although H2B K34ub histone octamers were assembled into nucleosomes on 5S DNA, hexasome formation was much more pronounced for both symmetrically- and asymmetrically modified H2B K34ub 5S nucleosomes when non-specific (plasmid DNA) or specific (NAP1) histone acceptors was added to the assembly (Figure 6D and E). Interestingly, H2B K120ub also significantly promoted H2A/H2B dimer eviction from 5S-nucleosomes, which was facilitated by higher temperature, ionic conditions or excess of competitor plasmid DNA (Figure 6B and C). These results suggest that H2Bub-mediated

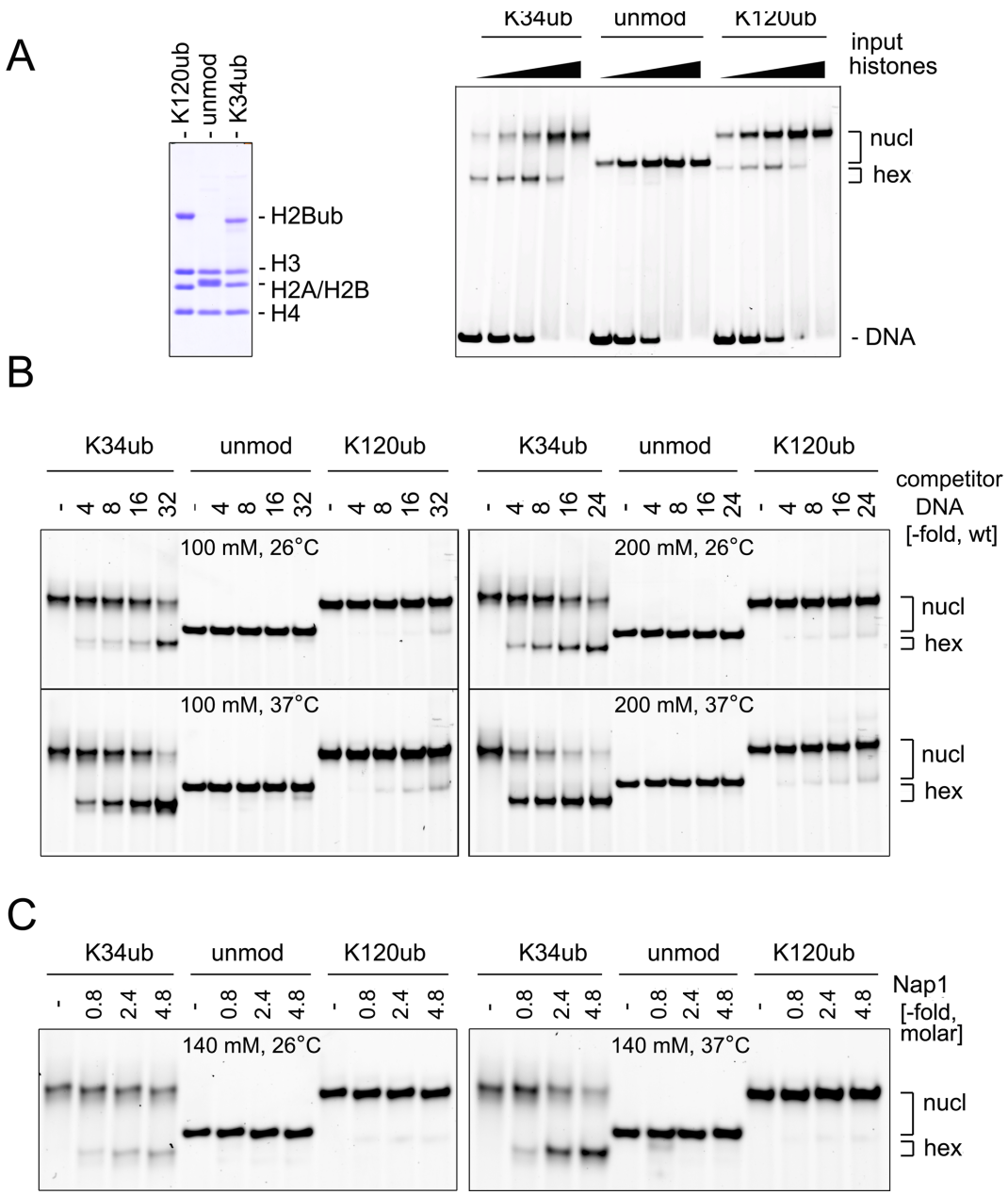

Figure 5. Stability of H2B K34ub- versus H2B K120ub-nucleosomes. (**A**) Left panel: SDS-PAGE of H2B K120ub and K34ub histone octamers. Right panel: unmodified and H2B K120ub/ K34ub nucleosomes assembled on 147 bp 601 DNA. (**B**) Nucleosomes assembled on 147 bp 601 DNA were post-assembly incubated with competitor DNA for 2.5 h at indicated temperature/ionic conditions. (**C**) Nucleosomes assembled on 147 bp 601 DNA were post-assembly incubated with NAP1 for 2.5 h at 26/37°C in a buffer containing at 140 mM NaCl.

increase of nucleosome dynamics as well as H2A/H2B dimer eviction may play a more significant role on the physiological chromatin templates *in vivo* (see 'Discussion' section), which warrants future studies.

DISCUSSION

Here, we show that the H2B K34ub nucleosome structure is mostly intact and binding of linker histone H1 is not obstructed, consistent with a previous cryo-EM study (48) We further show that H2B K34ub effectively destabilizes the symmetrically- and asymmetrically modified nucleosomes by evicting one H2A/H2B dimer. This function is facilitated by DNA supercoiling stress and histone chaperone NAP1. H2B K34ub also increases intrinsic nucleosome dynamics, which may facilitate transient access and/or movement of DNA binding factors on chromatin. These results provide the molecular basis for a function of H2BK34ub in transcription regulation (9,10,67,68).

The presence of hexasomes at transcriptionally active chromatin loci was first described nearly 40 years ago (85,89). Later studies have further confirmed involvement of hexasomes in multitude vital chromatin-templated processes including replication, DNA recombination, DNA

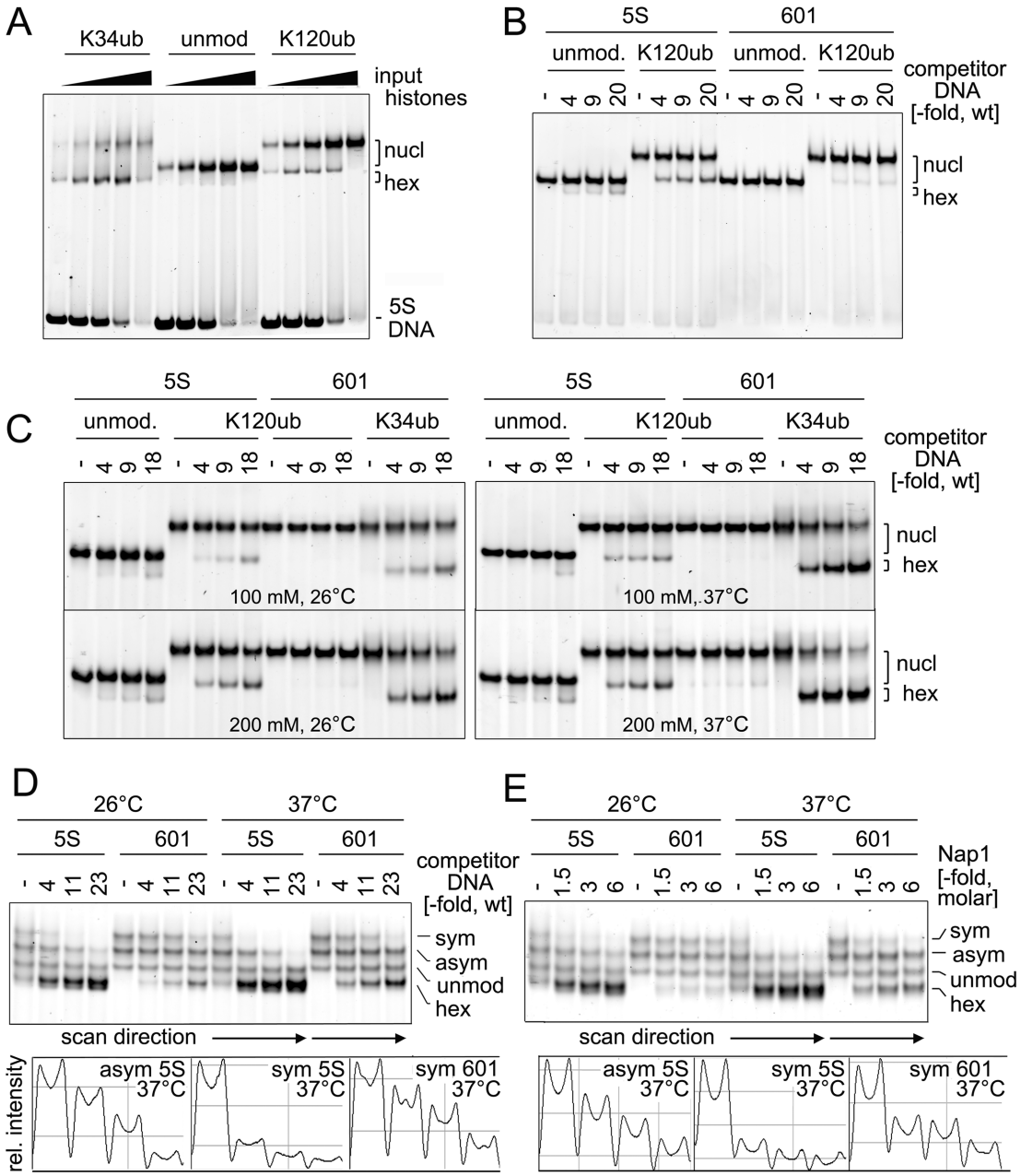

Figure 6. Effects of underlying DNA sequence on stability of H2B K120ub and K34ub nucleosomes. (**A**) Nucleosomes assembled on 146 bp 5S DNA. (**B**) Assembled nucleosomes were incubated with competitor DNA for 2.5 h at 26°C in a buffer containing at 200 mM NaCl. (**C**) Nucleosomes incubated with competitor DNA for 2.5 h at indicated temperature/ ionic conditions. (**D** and **E**) Nucleosomes assembled with unmodified or H2B K34ub histones, or their 1/0.57 mixture, were post-assembly incubated for 2.5 h at 26 or 37°C with (D) competitor DNA in a buffer containing at 100 mM NaCl or (E) NAP1 in a buffer containing at 150 mM NaCl. Densitometry tracing of indicated gel lanes is shown at the bottom of gel images.

repair and all stages of transcription (9,13,69,70). Recent CHIP-exonuclease and MNase-based assays demonstrate widespread distribution of hexasomes and other partially assembled nucleosomes structures in budding yeast *in vivo* (94). Hexasome is also reported as transcription intermediates on active chromatin *in vivo* (71). Hexasome formation *in vivo* may be a result of intensive histone H2A-H2B exchange by histone chaperones (e.g. NAP1 and FACT) (38,67,72–74), RNA or DNA polymerase movement (75,75,76) and chromatin remodeling activities (77,78). These mechanisms are not mutually exclusive. It has been reported that passage of RNA pol II through chromatin is accompanied by transient displacement and re-association of H2A-H2B dimers (9,10), which is facilitated by FACT activity (67,68). In fact, RNA Pol II requires hexasome formation with the H2A/H2B dimer at promoter-distal orientation at entry point and at promoter-proximal orientation at exit to prevent RNA Pol II stalling (38,75). The precisely orchestrated H2A/H2B dimer disassembly

Table 1. DNA was assembled with increasing total amount of unmodified histones, H2B K34 ubiquitylated histones, and their mixture to produce unmodified, symmetrically and asymmetrically modified nucleosomes

Input histones									
Modified/ Unmodified	3/1			1 /1			0.33/ 1		
Experimental value:									
Symmetric	0.6	0.7	1.2	0.1	0.1	0.2	0.0	0.0	0.0
Asymmetric	1.8	2.0	2.0	0.8	0.7	0.7	0.2	0.2	0.1
Unmodified	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Expected value (calculated based on statistical probability):									
Symmetric	9.0			1.0			0.11		
Asymmetric	6.0			2.0			0.67		
Unmodified	1.0			1.0			1.0		

These samples were analyzed on native PAGE (see Figure 2A). Top part of the table shows quantification of the image in Figure 2A; the bottom part shows predicted values that are based on statistical probability of assembly product using 3:1, 1:1 or 0.33:1 ratios of modified versus unmodified histone octamers as indicated on top. The predicted values assume that H2B K34ub does not affect binding of the second H2A-H2B dimer. The values in the table are normalized to the amounts of unmodified nucleosomes assembled in each reaction (top), which was arbitrarily assigned as 1. The large discrepancies between the ratios measured from the assembled nucleosomes and prediction clearly indicated that H2B K34ub interferes with the binding of the second H2A-H2B dimer.

and re-association process highlights significant regulatory role of hexasome formation in transcription regulation.

Our study shows that H2B K34ub by the MSL complex represents a new mechanism that promotes hexasome formation and this function is further enhanced by DNA torsional tension and histone chaperon NAP1. Since Pol II traversing through the nucleosome requires tightly coordinated displacement and reassembly of H2A-H2B dimers (9) and at the same time, generate positive or negative DNA torsional tensions (61,79,80), we envision a positive feedback regulation between the MSL complex and RNA Pol-II in productive transcription. In this model, the PAF1 complex recruits and stabilizes the MSL complex on chromatin (45), which in turn deposits H2B K34ub. H2B K34ub destabilizes nucleosomes and increases dynamic H2A/H2B dimer exchange with the help of histone chaperone NAP1. The increase of Pol II transcription produces additional DNA supercoiling stress, which further enhances H2Bub-mediated hexasome formation (Figure 2B). This model is consistent with our previous observation that MSL binding has genome-wide correlation with high processivity of RNA Pol II *in vivo* (45). Notably, H2B K34ub not only promotes hexasome formation, but also significantly increases lability of the H2A/H2B dimer in the asymmetrically modified nucleosomes. We find that binding of the second modified or unmodified H2A-H2B dimer to H2B K34ub hexasomes is much less efficient (Figure 2). Thus, H2B K34 ubiquitylation is able to 'preserve' the hexasome state, preventing it from subsequent assembly into full nucleosome. This is potentially important for maintaining an 'open' and functionally active chromatin state, which further facilitates activity of DNA-bound enzymes such as RNA polymerases. Future studies on MSL-mediated hexasome formation *in vivo* will shed light on this.

One interesting finding in our study is that H2B K34ub greatly increases dynamics of nucleosomes as exemplified by the 'extended' MNase cleavage pattern as well as increased heterogeneity of electrophoretic mobility at higher temperature or salt condition (Figure 3). These results suggest that H2B K34ub nucleosomes may promote spontaneous unwrapping of nucleosomal DNA (81–84). Given location of H2B K34 between two DNA gyres, it is possible that H2B K34ub increases the gaping motion of nucleosomes, i.e. a slow spontaneous hinge-like motion in the direction perpendicular to DNA plane (13,85), which remains to be experimentally evaluated. Nonetheless, increase of spontaneous nucleosome fluctuations or transient DNA exposure is one of the major mechanisms that regulate access of transcription factors *in vivo* (13,83,86,87), which also contributes to overall transcription activities. The function of H2B K34ub in nucleosome dynamics is reminiscent of other histone PTMs at nucleosome entry or dyad axis such as H3K56ac, H3Y41phos as well as H3K115ac, H3K122ac (15,17,24). It would be interesting to test whether H2B K34ub functionally cooperate with these histone PTMs in promoting transcription or other chromatin based activities. Notably, H2B K34ub resides within a highly conserved HBR (H2B repression) domain (88,89), which includes residues 30–37 in yeast H2B (equivalent to residues 27–34 in Xenopus H2B, used in this study). The HBR domain functions to repress 10% of the yeast genome (88,90). A previous study shows that mutations in the HBR domain significantly compromise nucleosome assembly efficiency on 5S DNA (91). They also affect interactions between nucleosomes and histone chaperone FACT (91,92). While it

remains to be determined whether H2B K34ub is conserved in yeast, our study suggests that the function of HBR in facilitating cooperative incorporation of the H2A/H2B dimer could be modulated by histone ubiquitylation, which warrants future studies.

At last, we show that in addition to H2B K34ub, H2B K120ub also plays a role in regulation of nucleosome stability and dynamic, although its effect is much modest in comparison. Previous studies show that ubiquitylation at H2B K120 had only marginal effects on stability of the 601 nucleosomes *in vitro* (64,65) despite its function in promoting Pol II transcription (67,93) as well as nucleosome reassembly (37) and stabilization *in vivo* (94). Using a more physiologically relevant 5S DNA template, we show that H2B K120ub has a modest, but significant, effect on nucleosome stability *in vitro*, which leads to increase of H2A/H2B dimer eviction (Figure 5). Notably, our previous study shows that H2B K120ub is better at promoting H3 K4me by the MLL1 complex than H2B K34ub (Wu *et al.*, (43)). Since here we show that H2B K120ub has much more modest effect on nucleosome stability than H2B K34ub, it suggests that regulations of nucleosome stability and histone H3K4me by H2B ubiquitylation are probably decoupled. This is consistent with the fact that MLL1 has similar activity on nucleosomal and tetrasomal substrates (unpublished observation) as well as lack of evidence that histone H2A-H2B chaperones, such as NAP1, have direct function in stimulating H3K4me despite their regulation of H2A-H2B dynamics. Taken together, our study reveals a general role of H2B ubiquitylation in nucleosome regulation and its potential implication in transcription activation.

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