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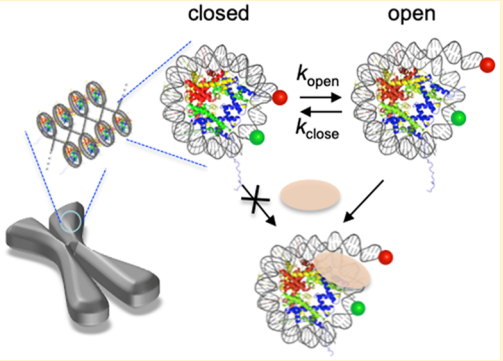
Physical Chemistry of Epigenetics: Single-Molecule Investigations

Tae-Hee Lee*

Department of Chemistry, The Pennsylvania State University, University Park 16803, Pennsylvania, United States

ABSTRACT: The nucleosome is the fundamental building block of the eukaryotic genome, composed of an ∼147 base-pair DNA fragment wrapping around an octameric histone protein core. DNA and histone proteins are targets of enzymatic chemical modifications that serve as signals for gene regulation. These modifications are often referred to as epigenetic modifications that govern gene activities without altering the DNA sequence. Although the term epigenetics initially required inheritability, it now frequently includes noninherited histone modifications associated with gene regulation. Important epigenetic modifications for healthy cell growth and proliferation include DNA methylation, histone acetylation, methylation, phosphorylation, ubiquitination, and SUMOylation (SUMO = Small Ubiquitin-like Modifier). Our research focuses on the biophysical roles of these modifications in altering the structure and structural dynamics of the nucleosome and their implications in gene regulation mechanisms. As the changes are subtle and complex, we employ various single-molecule fluorescence approaches for their investigations. Our investigations revealed that these modifications induce changes in the structure and structural dynamics of the nucleosome and their thermodynamic and kinetic stabilities. We also suggested the implications of these changes in gene regulation mechanisms that are the foci of our current and future research.



INTRODUCTION

DNA in a eukaryotic cell is packaged in the nucleus. The level of compaction is extreme at 3 billion base pairs (bp) within 0.7 fL in a HeLa (HeLa, Henrietta Lacks) cell nucleus,[1] equivalent to a string of 200 million miles packaged in a baseball. The sequence of DNA in a genome carries mainly the information on the amino acid sequence of proteins that the cell can produce. As the proteins are the main workhorses of cellular functions, the sequence of the entire genome essentially dictates the birth, life, and death of the cell. For a cell to produce a protein molecule, it transcribes the corresponding gene to mRNA that will be translated into the protein, constituting the central dogma of biology. How to regulate transcription is, therefore, arguably the most important point of implementing and controlling the cellular functions and is a very complex process often composed of multiple layers. Near the top of the layers, there is regulation of the structure and structural flexibility of the nucleosome that is the basic building block of chromatin and the most fundamental packing unit of a eukaryotic genome (Figure 1).[2−5] Chromatin is the protein−nucleic acid complex mainly composed of histone and DNA that forms a nucleosome core particle, an ∼147 bp DNA fragment wrapping around an octameric histone protein core. The basis of DNA packaging in the nucleosome is the electrostatic interactions between DNA, which is acidic, and the histone core that contains ∼20% of basic residues. Nucleosomes form a beads-on-a-string structure that folds into a fiber and eventually compacts into a chromosome.[6,7] Therefore, how to control the structure and flexibility of the nucleosome and the thermodynamics and kinetics of nucleosome assembly and disassembly are at the core of gene regulation mechanisms. In most cases, these regulatory activities involve chemical modifications of DNA and various proteins.[6,8−14]

DNA and histone proteins are targets for enzymatic chemical modifications including CpG methylation, histone lysine acetylation, arginine/lysine methylation, serine/threonine/tyrosine phosphorylation, and lysine ubiquitination/SUMOylation (SUMO = Small Ubiquitin-like Modifier; Figure 2).[6,8−18] Histone variants can replace a part of the histone core, resulting in a modified nucleosome core particle.[19−24] Most of these modifications serve as a gene regulatory signal, dictating, for example, whether a gene should be activated for transcription, whether a gene should be kept transcriptionally active for an elongated period of time, whether a gene should be temporarily repressed, and which part of the chromosome should be permanently silenced at the developmental stage. Some of these modifications have a direct impact on the overall structure of chromatin, resulting in chromatin remodeling.[25−30] This layer of gene regulation and restructuring is via chemical modifications on top of the genetics dictated by the DNA sequence, hence, named epigenetics.[31] The term epigenetics initially required inheritability and now often includes other changes that control gene activities without altering the DNA sequence.[31]

Some epigenetic modifications can function mainly by recruiting enzymes for cascading biochemical reactions, and some may function by altering the structure and structural

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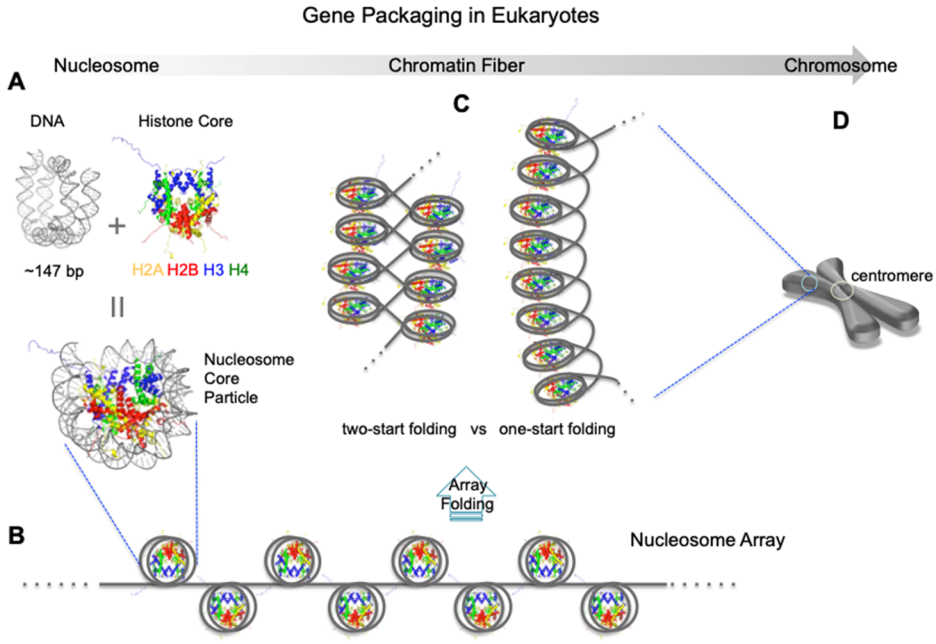

Figure 1. Hierarchical gene packaging in eukaryotes. (A) Nucleosomes (PDB ID: 1AOI) are linked in (B) an array format that folds into (C) chromatin fiber and eventually into (D) chromosome. The chromatin fiber structure is widely hypothesized to be either two- or one-start helically folded. For cell division, chromosomes are replicated and are organized into a distinct shape with a centromere that connects the original and the replicated chromatids.

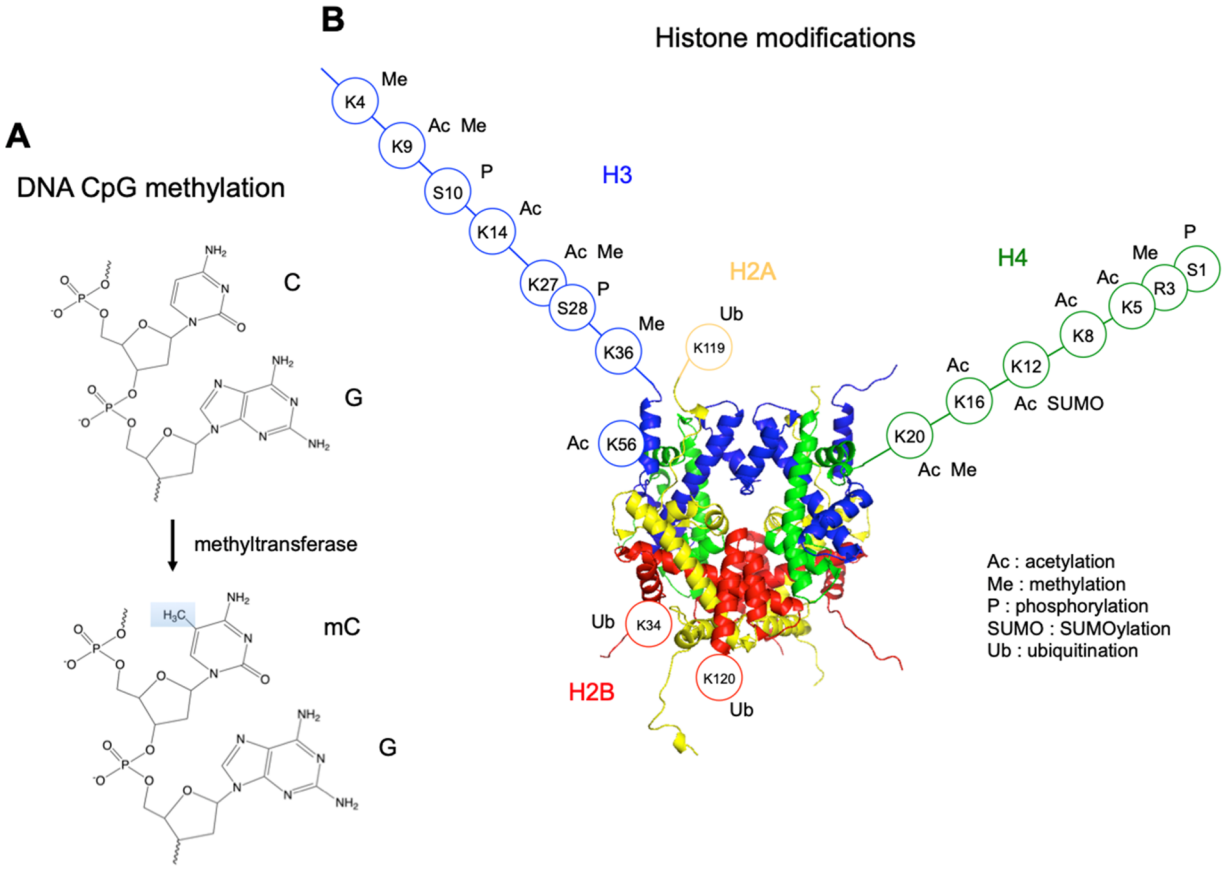

Figure 2. Epigenetic DNA and histone modifications. (A) DNA CpG methylation is to methylate the C5 position of the cytosine base in a CG dinucleotide sequence and is performed by methyltransferase enzymes. (B) Various histone modifications that are associated with gene regulation activities. The histone structure is from PDB (ID: 1AOI). The modifications are mostly concentrated in the H3 and H4 N-terminal tails.

flexibility of the nucleosome and chromatin. This review focuses on our publications regarding the effects of some important epigenetic modifications on the structure and flexibility of the nucleosome and the thermodynamics and

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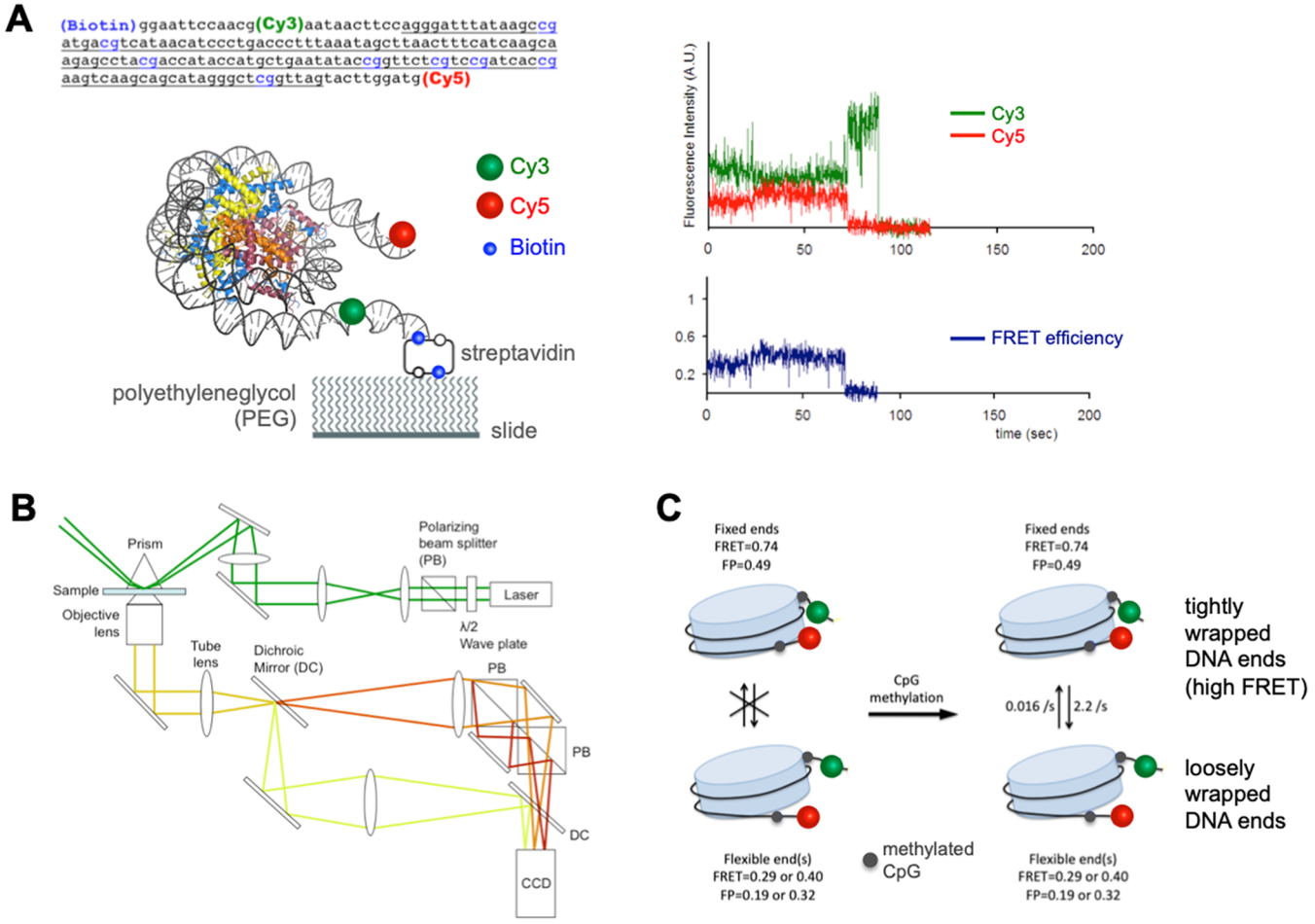

Figure 3. CpG methylation induces structure and structural changes of the nucleosome termini.[38] (A) Experimental setup showing the nucleosomal DNA sequence (5S rDNA) and a cartoon of the assembled nucleosome labeled with a FRET pair (Cy3 and Cy5). The nucleosome has a biotin at one end that is conjugated to streptavidin immobilized on a poly(ethylene glycol) passivated glass slide. (B) Microscope setup showing the measurement scheme of FRET acceptor emission polarization. A prism introduces a totally internally reflected donor excitation laser beam to the surface of the glass slide with immobilized nucleosomes. The emission is collected through a long-pass filter (omitted) to filter out the laser excitation and divided into two spectral regions with a dichroic mirror (DC). The donor emission is introduced to a relay lens that focuses and projects the wide-field image on an EMCCD camera. The acceptor emission is introduced to a relay lens that either focuses and projects directly to the EMCCD camera or projects through another color or polarization filter (PB) to further split the emission. The current setup images the donor emission and the parallel and perpendicularly polarized acceptor emissions, dividing the EMCCD camera chip into three spatial regions. Without the PB along the acceptor emission path, the setup images the donor and acceptor emissions only. The time trajectories of the fluorescence emission intensities from one donor−acceptor pair are shown in (A). (C) On the basis of the observations, it was found that CpG methylation induces excursions to a tightly wrapped nucleosome structure, validating a hypothesis that CpG methylation makes the nucleosome structure more difficult to invade, thereby contributing to gene repression and silencing. Adapted with permission from ref 38. Copyright 2010 American Chemical Society.

kinetics of nucleosome assembly and disassembly and how these effects may be implicated in gene regulation mechanisms. The size of the nucleosome is ∼220 kDa, ∼10 nm wide and ∼8 nm thick, which is too small for direct observation with optical microscopy techniques and too large to "see" with NMR techniques. Moreover, the structural dynamics of the nucleosome in the context of protein binding, chromatin remodeling, and transcription is so complex that their investigation is nearly impossible with ensemble-averaging techniques. Our approach is based on single-molecule fluorescence measurements, circumventing these limitations. Single-molecule spectroscopy and microscopy provide an efficient means to avoid ensemble averaging, thereby enabling investigations on a complex system that cannot be easily synchronized.[32−37] We have utilized various single-molecule techniques such as polarization specific fluorescence resonance energy transfer (FRET),[38,39] two- and three-color FRET,[40−43] photon-by-photon fluorescence correlation,[44,45] and maximum likelihood estimation.[45−48] By utilizing single-molecule methods customized for each problem, we elucidated several important aspects of epigenetics regulating the thermodynamic and kinetic stabilities of the nucleosome in various contexts. Our systems allow for clean and straightforward measurements without any interference from unknown factors in a cellular environment, enabling in-depth mechanistic studies.

DNA Methylation Increases Nucleosome Compaction and Rigidity. Arguably the most important DNA modification is CpG methylation (Figure 2A),[8,11,49] as it is involved in proper organ development and associated with tumor suppressor regulation. Hyper-methylation of CpG in the promotors of various tumor suppressor genes has been reported.[50−53] CpG methylation is methylation at the C5

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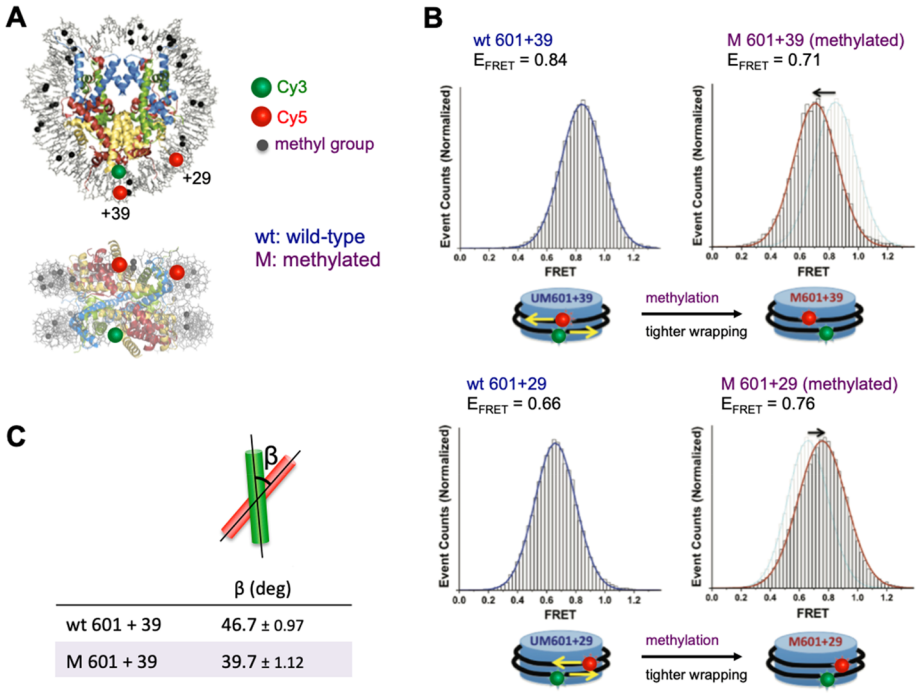

Figure 4. CpG methylation induces structural changes of DNA in the internal regions of the nucleosome.[39] (A) Two different FRET pair locations (acceptor at the +39th or +29th nucleotide from the entry site of the nucleosome) were used to test the hypothesis that CpG methylation tightens DNA wrapping and induces DNA gyre shifting accordingly. (B) The nucleosomes with the 601 DNA sequence labeled with a FRET pair at the +39 and +29 positions, namely, 601 + 39 (upper) and 601 + 29 (lower), respectively, show tighter wrapping of DNA upon CpG methylation. (C) Results from polarization-dependent (parallel and perpendicular) FRET acceptor emission measurements can be converted to the interdipole angle (β) between the donor (emission) and acceptor (absorption). The values in the table clearly show a DNA topology change due to gyre shifting upon CpG methylation. Adapted with permission from ref 39. Copyright 2012 American Chemical Society.

position of the cytosine nucleotide in a CpG dinucleotide step (note: "p" denotes the phosphodiester bond connecting the two nucleotides C and G). Over 70% of CpG cytosines in mammalian cells are methylated.[10] CpG methylation is performed by DNA methyltransferases and is an inheritable epigenetic mark essential for proper development, healthy growth, and proliferation of cells.[54]

CpG methylation is associated mostly with gene repression.[55−60] A methyl group attached to a cytosine base may exert a considerable physicochemical force, as CpG methylation takes place in a highly concentrated manner in the regions called "CpG islands".[61] It has been shown that the majority of human promoters align with CpG islands, supporting that CpG methylation may have direct biophysical roles in gene regulation.[61,62]

The mechanism of how CpG methylation represses gene expression remains largely unknown. A group of proteins called methyl DNA binding proteins or methyl CpG binding proteins contain methyl-CpG-binding domain (MBD) and recognizes methylated CpG.[63,64] On the one hand, these enzymes bind methylated CpG regions and trigger cascading reactions to eventually result in chromatin compaction and transcription repression. On the other hand, we reported that CpG methylation increases the rigidity and compaction of the nucleosome and that CpG methylated DNA facilitates nucleosome assembly.[38,39,65,66]

We utilized polarization specific single-molecule FRET (smFRET) to investigate the changes in the DNA structure and flexibility of the nucleosome with and without CpG methylation (Figure 3).[38] FRET is a resonance energy transfer process between two fluorophores and can be observed at a single-molecule level that is referred to as smFRET.[40] It can be modeled with dipole−dipole coupling whose strength depends on several factors including the angular alignment, distance, and absorption-emission spectra of the fluorophores. A stronger spectral overlap between the donor emission and the acceptor absorption results in a higher FRET efficiency. When the fluorophores can rotate freely, the angular dependence is averaged during a typical FRET measurement on a microsecond or longer time scale. In such a case, the FRET efficiency depends on the reciprocal of the sixth power of the distance between the fluorophores as in $FRET\ efficiency = \frac{1}{1+\left(\frac{r}{R_0}\right)^6}$, where r is the distance between the fluorophores, and R_0 is a constant integrating the other factors and is often referred to as Förster radius. The Förster radii of widely used FRET pairs with a moderate spectral overlap such as Cy3/Cy5, Cy3/ATTO647N, and Fluorescein/Rhodamine 6G are suitable for investigating distances and distance changes in the 1−10 nm range. When the fluorophores have restricted rotational motions or they are labeled rigidly at an entity that can undergo a restricted rotational motion, the FRET acceptor fluorescence polarization can be a good indicator of how freely the fluorophores can rotate. By utilizing FRET efficiency and acceptor polarization, we probed the structure and flexibility changes of the nucleosome induced by CpG methylation. We used Cy3 and Cy5 for the FRET pair. The photophysical properties of Cy3 and Cy5 have been very well-characterized. These fluorophores stack with DNA bases when labeled to DNA ends, which restricts their rotational motions, thereby suppressing their photophysical and chemical fluctuations

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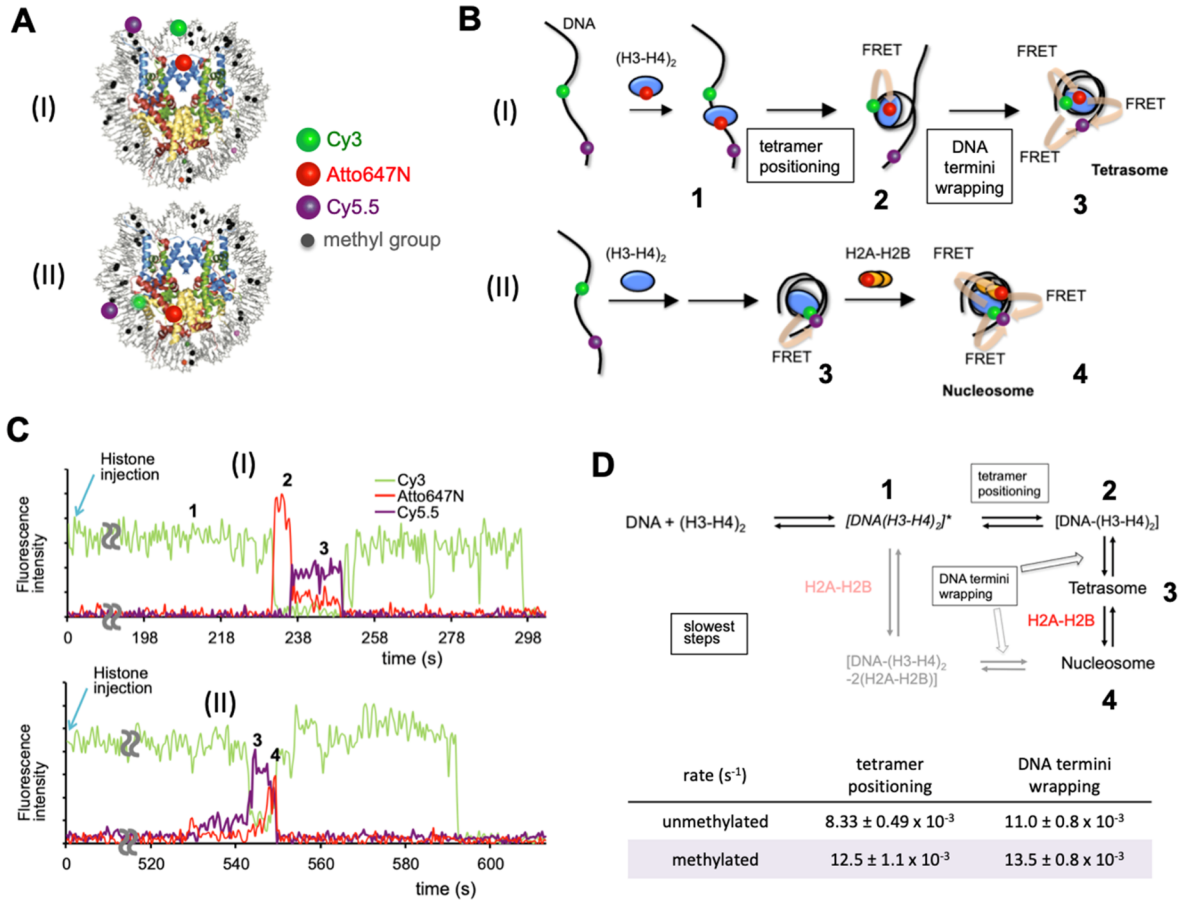

Figure 5. Nucleosome assembly kinetics and CpG methylation effects.[66] (A) Three-color smFRET was used to monitor the assembly in two subprocesses. The setup (I) was used to monitor tetramer binding and tetrasome formation and (II) was used to monitor dimer binding and nucleosome formation. These two subprocesses are depicted in (B). (C) Representative three-color FRET intensity time trajectories for the subprocesses (I), upper, and (II), lower. The microscope setup is similar to that in Figure 3B, except that the polarizing beam block (PB) is replaced with a dichroic mirror that separates Atto647N emission from Cy 5.5 emission. (D) The rate constants measured from the smFRET measurements revealed that nucleosome assembly takes place dominantly via tetramer formation and subsequent dimer binding (black lines and letters) rather than via octamer binding and DNA wrapping (gray lines and letters) at a 9:1 ratio. The slowest steps are tetramer positioning and DNA wrapping steps, in both of which CpG methylated DNA facilitates assembly, validating our hypothesis. This research was originally published in the *Journal of Biological Chemistry*. Ju Yeon Lee, Jaehyoun Lee, Hongjun Yue, and Tae- Hee Lee, Dynamics of nucleosome assembly and effects of DNA methylation. *J. Biol. Chem.* **2015**, *290*, 4291−4303. © the American Society for Biochemistry and Molecular Biology.

and perturbations.[67,68] Cy3 and Cy5 labeled in the middle of a DNA sequence by a commercial source (e.g., Integrated DNA Technologies, Inc.) are doubly anchored at both ends along the phosphate backbone of DNA, forming a bulgelike structure and, therefore, incapable of free rotation. For protein labeling, fluorophore−protein interaction for photophysical and chemical stabilization is not always possible, and therefore, other fluorophores with higher inherent stability and intensity (e.g., ATTO647N) may be preferred.

The fluorescence microscope setup is standard and described well in previous reports.[38,69] The setup splits the fluorescence emission into the donor and acceptor spectral regions and the acceptor emission further into the two polarization states perpendicular to each other (Figure 3). The FRET fluorophores were labeled rigidly on the nucleosomal DNA ends, and therefore, their rotation reflects the rotational freedom of the nucleosomal DNA ends. We found that CpG methylated nucleosomes show excursions to a tightly wrapped state, suggesting that CpG methylation induces more complete wrapping of the nucleosomal DNA (Figure 3C). Furthermore, the high FRET state shows a very high acceptor fluorescence polarization, strongly supporting that the DNA in the wrapped state is very rigid. These results indicate that CpG methylation induces tight wrapping and rigid structure of the nucleosome. These changes imply that CpG methylation reduces the bendability and twistability (measured by bending and twisting force constants)[5] of the nucleosomal DNA termini, restricting their conformational space in the free form, thereby stabilizing the nucleosome. This conclusion strongly supports the role of CpG methylation in repressing transcription by strengthening nucleosome compaction and consequently inhibiting protein binding to the DNA. To further confirm this result deeper inside of the nucleosome, we probed the changes in the internal DNA regions of the nucleosome (Figure 4).

The setup is similar to the one described in Figure 3 but with the FRET pair labeled at DNA in internal regions of the nucleosome.[39] The FRET efficiency changes before and after CpG methylation in the cases with two different labeling positions consistently indicate that the DNA wrapping becomes tighter upon methylation (Figure 4B). Both of the results in Figures 3 and 4 support that CpG methylation induces compaction and rigidity of the nucleosome. We

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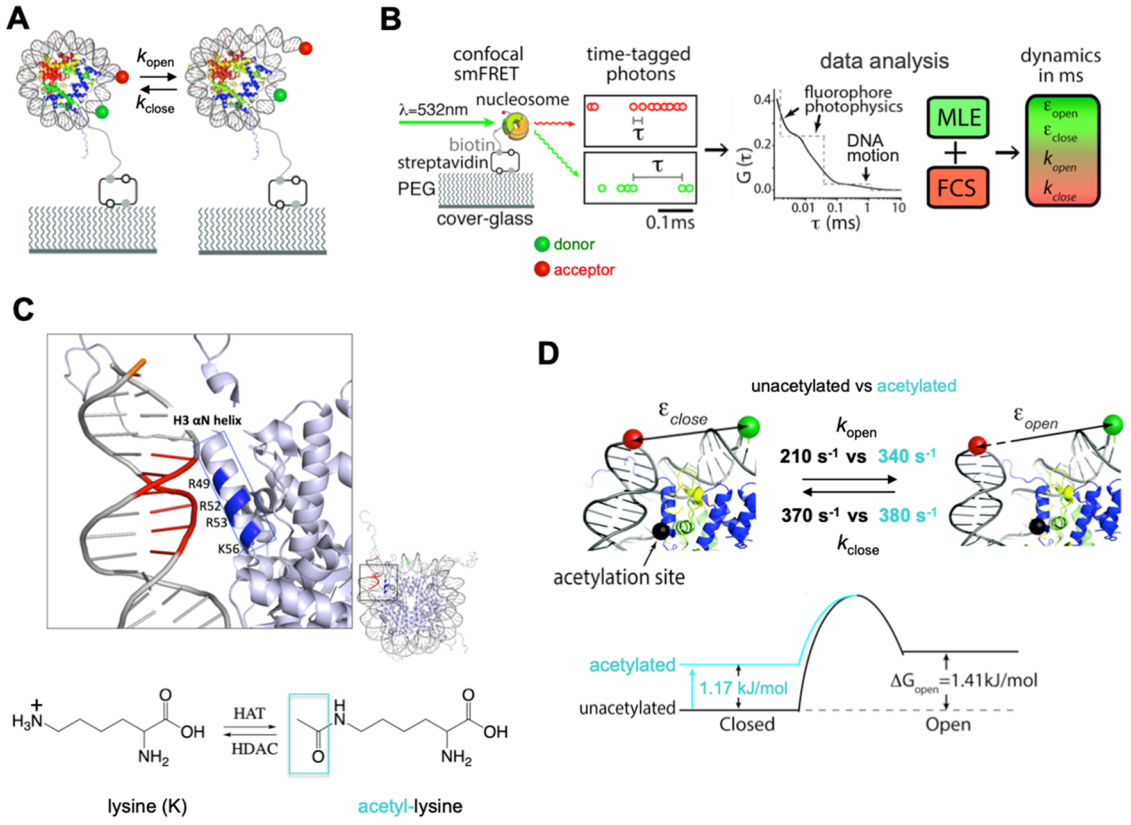

Figure 6. Fast nucleosomal DNA opening and closing motions and H3K56 acetylation effects.[45,47] (A) The nucleosome is labeled with a FRET pair that reports the DNA termini opening and closing motions. (B) Measurement and analysis scheme. FRET emission is collected on a microscope in a confocal geometry with two units of avalanche photodiode for donor and acceptor, respectively. The photon arrival times at the detectors are recorded at a 50 ns time resolution. MLE was employed to extract the open- and closed-state FRET efficiencies, ε_{open} and ε_{close}, respectively, from the photon sequences. The rate constants for the opening and closing motions, k_{open} and k_{close}, respectively, were obtained by combining FCS. The MLE-FCS method reports the four parameters at a high confidence level. (C) The location of H3K56 and the reaction scheme of charge neutralization of a lysine residue by acetylation. (D) The results show that the hypothesized spontaneous DNA opening motion exists with a $\Delta G_{opening}$ value of 1.41 kJ/mol and that the motion is affected by H3K56 acetylation (H3K56ac). Adapted with permission from ref 45 and from ref 47 (Copyright 2015 American Chemical Society).

confirmed the results further by testing if tighter wrapping accompanies a DNA topology change based on the FRET acceptor anisotropy measurements that result in the interdipole angle (Figure 4C).[39]

In addition to the increased bendability and twistability, these results suggest that CpG methylation also induces static bending and twisting[5] of the DNA favorable for nucleosome formation, although the effect should depend on the locations of CpG dinucleotides. The nucleosomal DNA sequence that we used in this investigation is derived from the Widom 601 sequence, a strong nucleosome positioning sequence that was selected in a large pool of random synthetic DNA.[70,71] The sequence contains 15 CpG sites, most of which has direct contact with the histone core, suggesting that the increased static bending and twisting of DNA should help strengthening DNA−histone interactions and nucleosome compaction.

Elucidating the sources of these changes would entail investigations on how the methylated CpG affects the local structures of the nucleosome, where DNA and histone interact so that one can precisely compute its energetic contribution to the nucleosome structure and its formation. However, homogeneously methylated nucleosomal DNA in a quantity required for a crystallographic investigation is currently inaccessible. While our single-molecule investigations reported the direct physical changes induced upon CpG methylation and their impact on the thermodynamic stability of the nucleosome, they would not reveal the effects on the kinetics of nucleosome assembly.

CpG Methylation Facilitates Nucleosome Assembly. Our next investigation was to test how CpG methylation affects the kinetics of nucleosome assembly.[66] The experimental setup is based on three-color smFRET as shown in Figure 5. The setup enables detection of histone binding in the context of DNA wrapping during nucleosome assembly. The microscope setup is similar to what is shown in Figure 3 with the polarization filter replaced with a spectral filter to separate the two acceptor emissions from each other.[66] Nucleosome assembly was mediated by a histone chaperone Nap1 and monitored in real time in a time-resolved manner.

Nucleosomes can be assembled in vitro by gradually decreasing the salt concentration from 2 M NaCl to a low salt concentration via dialysis.[72] It has been known that the core histone proteins form a stable octamer composed of two H2A−H2B heterodimers and one $(H3-H4)_2$ tetramer at 2 M NaCl, which does not need to bind DNA for charge neutralization. As the salt level decreases, the octameric histone core starts breaking down to the two heterodimers and a tetramer. A tetramer first binds DNA and becomes stabilized by neutralizing the charge, forming a stable "tetrasome" structure.[66] At a further decreased NaCl

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concentration, two dimers subsequently bind a tetrasome, forming a stable nucleosome core particle. This assembly method requires slow change of the concentration so that the system can be kept at equilibrium during the entire process.

Another way to assemble the nucleosome, which is more physiological than salt dialysis, is to use histone chaperone.[73,74] Histone chaperone is a group of proteins mainly characterized by their spatially concentrated acidic residues that can compete against DNA for histone binding. Histone chaperone disturbs and mediates DNA histone interactions and helps them reach their thermodynamic equilibrium at a physiological salt concentration.[75] Some histone chaperones work for both H2A−H2B and $(H3−H4)_2$, while some are specific to H2A−H2B or $(H3−H4)_2$. Nucleosome assembly protein 1 (Nap1) is a generic histone chaperone that works for both the histone dimer and tetramer and drives the mixture of DNA, histone dimer, and tetramer in the 1:2:1 stoichiometry to form the nucleosome structure.[75] We employed Nap1 to mediate nucleosome assembly and monitored the pre-steady-state kinetics in real time with three-color smFRET with and without CpG methylation (Figure 5).[66]

Our observation indicates that $(H3−H4)_2$ tetramer first binds DNA before H2A−H2B dimers bind the tetrasome for nucleosome assembly. According to our results, CpG methylation accelerates the tetrasome positioning and DNA termini wrapping (Figure 5D), while it inhibits and decelerates proper incorporation of H2A−H2B. Inhibited H2A−H2B incorporation is due to facilitated random binding of the dimer to DNA. As the tetrasome formation and DNA termini wrapping steps are the slowest steps of nucleosome assembly, accelerated tetrasome formation leads to expedited nucleosome assembly overall. This change validates the hypothesis that the increased rigidity of nucleosomal DNA facilitates nucleosome assembly by lowering the entropy cost of DNA wrapping. Combined with the structural changes induced upon CpG methylation, this result strongly suggests that CpG methylation helps compact genes tighter and faster, thereby casting a higher barrier for protein binding to DNA.

Nucleosomal DNA Termini Opening Dynamics and the Effects of Histone H3K56 Acetylation. Next, we investigated nucleosomal DNA opening dynamics and the effects of acetylated H3K56 (H3K56ac).[45,47] Histone acetylation is typically associated with gene activation and active transcription.[23,76−78] On the one hand, enzyme complexes containing a bromodomain can recognize acetylated lysine with weak binding affinity at a level of a few micromolar dissociation constant.[79] On the other hand, lysine acetylation removes a positive charge from the histone core, weakening nucleosome compaction (Figure 6C). In particular, H3K56 is located at the entry and exit regions of the nucleosome, and thus its acetylation would exert a significant impact on the opening and closing motions of the nucleosomal DNA termini (Figure 6C). Nothing was known about the time scales of these motions prior to our reports,[45,47] although the rate constants of protein binding inside of a nucleosomal DNA sequence had been previously reported.[80]

There is a total of four arginine and lysine residues on the αN helix of H3 that interacts with the nucleosomal DNA termini (Figure 6C). The distance between the helix and the DNA backbone is 2−3 nm as estimated from crystal structures and FRET measurements.[45,71] Assuming the bulk relative permittivity of water (=78 at 25 °C), the electrostatic interaction energy of the four charge pairs is on the order of k_BT at 25 °C (4−6 vs 4.1×10^{-21} J), supporting the existence of spontaneous DNA opening motion and H3K56ac to function as a major regulator of the motion.

We first attempted to monitor the motion in the wild-type (wt) nucleosome based on a setup similar to those shown in Figures 3−5 that utilizes surface-immobilized nucleosomes and wide-field imaging with an electron-multiplying charge coupled device (EMCCD) camera. No such motion was visible down to a 10 ms temporal resolution, suggesting that it is much faster. We employed a confocal geometry that enabled collection of photon arrival times from individual nucleosomes at the cost of low labor efficiency for signal collection from one nucleosome at a time (Figure 6A,B).[45] The photon arrival times to the detectors contain the information on the dynamics that modulates the fluorescence signal including fluorescence decay, singlet−triplet equilibrium, fluorophore structural fluctuations, and nucleosomal dynamics. We used maximum likelihood estimation and fluorescence correlation to obtain the quantitative information on the nucleosomal dynamics.[45,47]

Maximum likelihood estimation (MLE) is a group of approaches to estimate the parameters characterizing dynamic changes of a system from their observable "emission". MLE methods had not been used to investigate single-molecule dynamics until recently.[46] Assuming a Markovian process,[81] a statistical model for the dynamic changes in a single-molecule FRET system can be constructed straightforwardly in most cases. A statistical model can be further developed into a likelihood function of the target parameters. Such likelihood functions have been reported for two- and three-state smFRET systems that emit fluorescence changes as a function of the parameters characterizing the dynamics.[82,83] Maximizing the likelihood function by systematically exploring the parameter space results in the optimum parameters that best represent the experimental observation. Several variations of the optimization algorithm are available, while none of them can guarantee that the convergence is at the global maximum. This is the inherent limit of any maximum likelihood estimation methods that cannot be overcome with the currently existing computing technologies and power.

By employing an MLE method, we found that the nucleosomal DNA (601 sequence + *X. laevis* histone) termini open once every 5 ms and close within 3 ms at 100 mM NaCl (Figure 6D).[47] These kinetics correspond to 1.41 kJ/mol less stable open state than the closed state. Because of the inherent limit of the method as described above, the precision of the estimation is low, and consequently some of the estimated parameters were widely distributed. To elevate the confidence level, we added fluorescence correlation to the analysis. The MLE analysis uses a likelihood function that takes the FRET efficiencies of the open and closed states and the rate constants between the two states. The precision of the FRET efficiencies is reasonably high, while that of the rate constants is low. On the basis of the MLE estimated FRET efficiencies of the open and closed states, we implemented fluorescence correlation spectroscopy (FCS) to extract the rate constants of the opening and closing motions at a high confidence level. This independent analysis dramatically improves the precision of the rate constants. Upon H3K56 acetylation, the opening frequency is increased to once every 3 ms, while the closing frequency is not affected. This change corresponds to 1.17 kJ/mol overall change in the activation energy of the motion (Figure 6). While this change is smaller than the thermal energy k_BT, it still leads to 30% longer dwell time in the open

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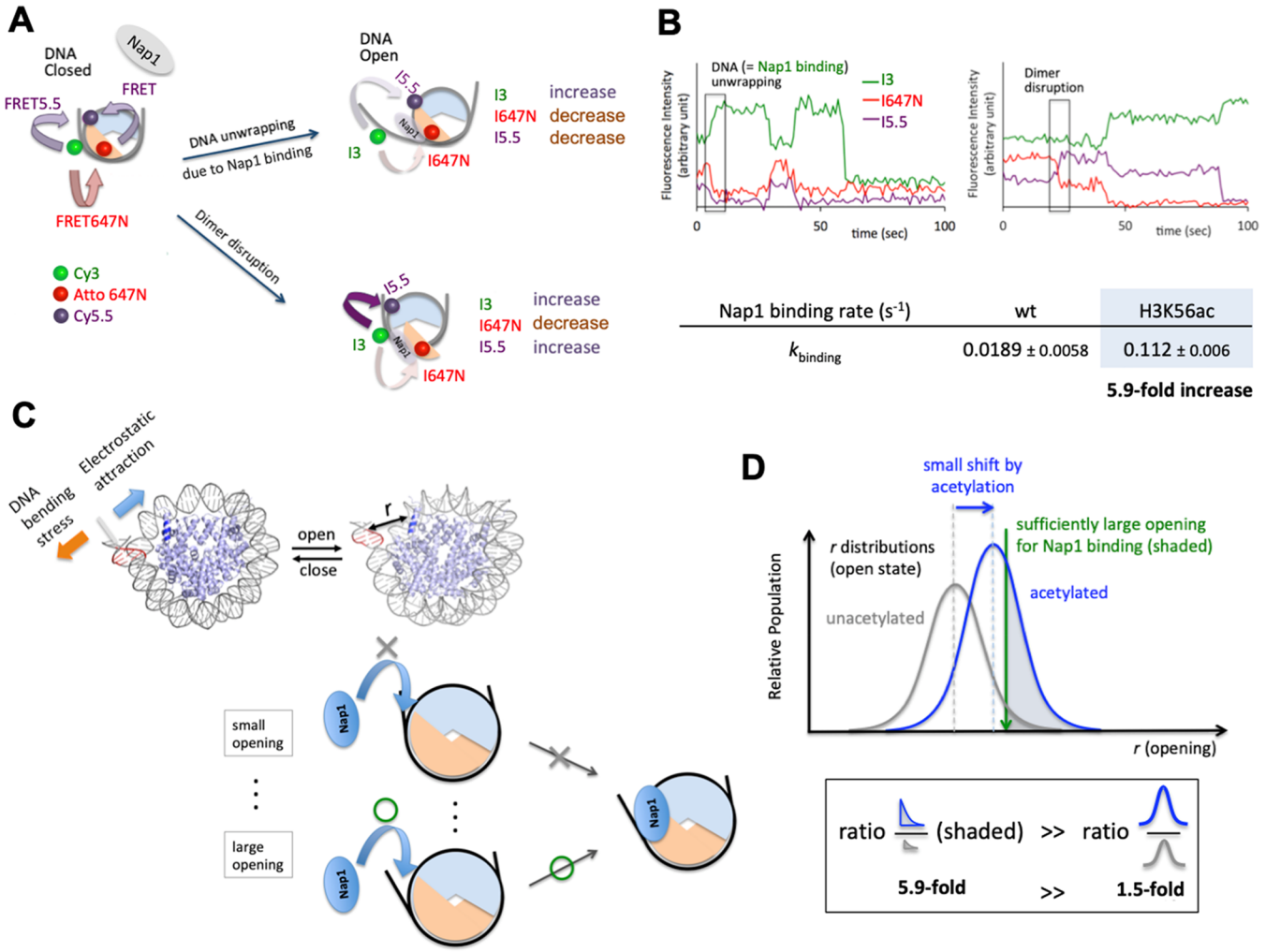

Figure 7. H3K56 acetylation (H3K56ac) dramatically facilitates protein binding in the nucleosome.[42,43] (A) Three-color FRET setup to monitor Nap1 binding with the histone core intact (upper path, DNA unwrapping). The data was filtered out when Nap1 binding takes place after the histone core is compromised (lower path, dimer disruption). (B) Typical intensity traces from the fluorophores that shows DNA unwrapping due to Nap1 binding (left) and dime disruption (right). DNA unwrapping traces were collected, and the Nap1 binding time was measured to show 5.9-fold increase in the acetylated nucleosome. (C) Proposed model for Nap1 binding. Nap1 binding can take place only when the spontaneous DNA opening is large enough to accommodate it. The spontaneous DNA opening motion is due to the balance between DNA bending stress and the electrostatic attraction between the DNA terminus and the H3 α-N helix. See detailed structure in Figure 6C. When one of the four positive charges of the H3 α-N helix is removed upon H3K56 acetylation, the DNA opening distance profile shifts a little bit toward a larger opening. This shift is shown in (D). For the change in the Nap1 binding efficiency upon H3K56ac, we must compare the shaded areas instead of the entire areas under the curves, according to the Nap1 binding model in (C). Because of a slight shift of the curve, the ratio becomes much more significant. Adapted with permission from refs 42 and 43. Copyright 2017 and 2019 American Chemical Society.

state at 100 mM NaCl (47% at 50 mM NaCl), as the stability difference between the open and closed states is only 1.41 kJ/mol. A similar level of change was also observed in the histone dimer motion upon H3K56ac,[48] further supporting that the spontaneous structural fluctuation of the nucleosome is facilitated upon H3K56 acetylation. However, this impact is unlikely sufficient to explain the strong effects of H3K56 acetylation on gene regulation. In the end, to properly evaluate the effect of H3K56ac on gene regulation, one should ask how significant the effect is on the efficiency of protein binding that requires nucleosomal DNA unwrapping.

Protein Binding and the Effects of Histone H3K56 Acetylation. To investigate the effect of H3K56ac on protein binding to the nucleosome, we used Nap1 as a model protein.[43] Nap1 is an excellent model protein, because it does not have any specificity in histone binding, and it binds the histone core only when DNA is unwrapped and the nucleosome structural integrity is compromised.[75] Therefore, the open state of the nucleosomal DNA can be stabilized by Nap1 binding, which is allowed only when the DNA is open at least transiently. We employed time-resolved three-color smFRET to monitor this long-term DNA unwrapping induced by Nap1 binding (Figure 7). The kinetics of this long-term DNA unwrapping directly represent Nap1 binding dynamics.[42]

We evaluated the binding kinetics of Nap1 with and without H3K56ac to find that the binding rate is increased by 5.9-fold upon H3K56 acetylation. This increase is far more significant than the mere 47% increase in the spontaneous opening rate of the nucleosome termini at the same salt level (50 mM NaCl).[47] To reconcile the discrepancy, we proposed a model for the enhancement of Nap1 binding upon H3K56 acetylation (Figure 7C). The model is based on a hypothesis that Nap1

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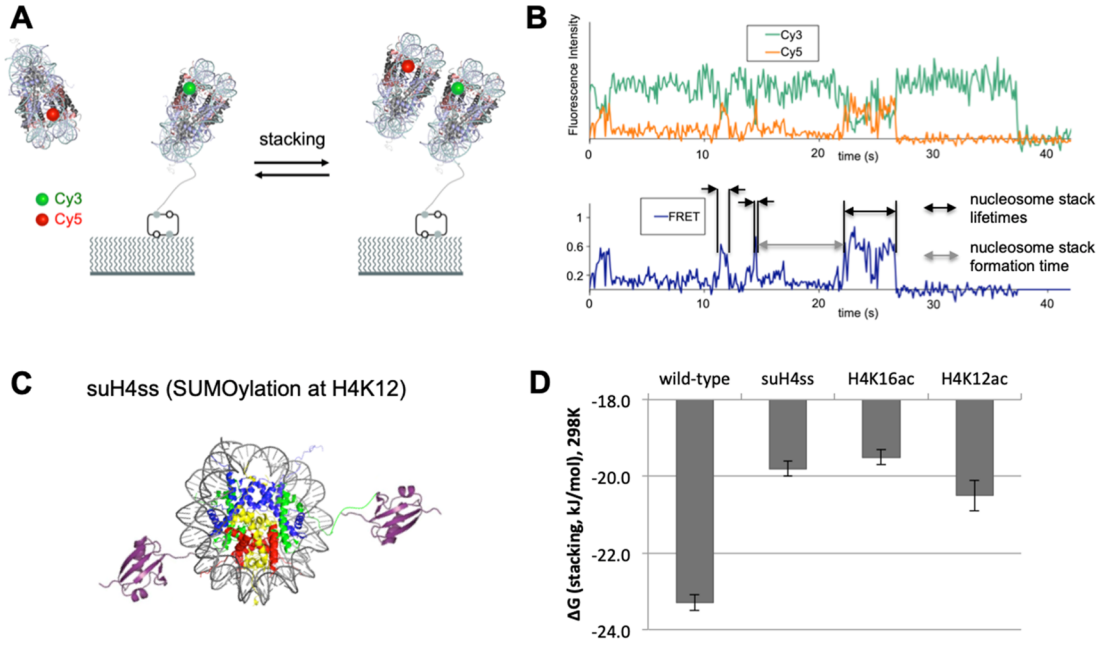

Figure 8. SUMOylation at H4K12 inhibits internucleosome stacking.[99] (A) Experimental setup to monitor dinucleosome stack formation and decomposition. (B) A representative fluorescence intensity and FRET traces show dinucleosome stack formation and decomposition. (C) A cartoon depicting an H4K12 SUMOylated (suH4ss) nucleosome. (D) The formation frequency and lifetime of the dinucleosome stacks as were measured from the FRET trajectories show that suH4ss inhibits nucleosome stacking as much as H4K12 and K16 acetylations (H4K16ac and H4K12ac, respectively). Further investigations on the rate constants suggested long-range interactions between two nucleosomes.

binding requires larger opening of DNA than the average transient opening. The opening kinetics are governed mainly by the bending potential of the DNA near the termini, which can be well-approximated with a wormlike chain model resulting in a quadratic function of the opening distance.[43,84] This is because the electrostatic interaction becomes negligible compared to the DNA bending energy at a 2−3 nm opening. Therefore, the Boltzmann distribution of the opening distance (r) makes the probability of reaching a long distance decreases as a function of $\exp(-r^2)$ (Figure 7D).[43] This means that a slight increase in the average opening due to H3K56ac will make a significant impact on the probability of reaching a long-distance opening. While the ratio between the entire areas under the blue and gray curves in Figure 7D is 47%, the ratio between the shaded areas can be far more significant. By using this model, we estimated that a long opening distance when the bending energy is ∼2 k_BT is reached six times more frequently upon H3K56ac. This model is further supported by a nearly constant success rate of binding in both the unacetylated and acetylated cases. Our measurements provide a self-consistent proof of the model for how spontaneous DNA opening motion sensitizes the nucleosome to the H3K56ac signal that amplifies 47% or 1.5-fold increase in the open-state dwell time to 5.9-fold increase in the Nap1 binding rate. This model is purely biophysical and does not require any unknown factor, yet it is sufficient to account for a large effect of histone acetylation. The mechanism is generally applicable to other protein binding and processing through the nucleosome such as RNA Polymerase II (Pol II) and chromatin remodelers. Future and currently undergoing investigations include the effects of histone acetylation on transcription activation by expediting protein binding and on transcription by facilitating Pol II translocation along nucleosomal DNA.[30,85]

Histone SUMOylation and Histone H4 Acetylation Inhibit Internucleosomal Stacking. The above modifications introduce chemically and physically small changes to the nucleosome, yet with significant biophysical impact on the structure and dynamics of the nucleosome. At the next level of chromatin compaction, nucleosomes linked in an array fold into a fiber. The hypotheses on the folding structure can be grouped into two: one-start and two-start helix folding structures (Figure 1C) whose formation depends on the linker length between two adjacent nucleosomes.[86−90] The structure of a tetranucleosome array with 30 bp linkers confirmed a two-start helix.[86] Linker histone H1 binds the linker region of a nucleosome array and contributes to chromatin compaction.[91,92] A hexamer nucleosome array with H1 bound also showed two-start folding structure.[89] However, as the folding conformation is a function of several parameters including the linker length and its variation, heterogeneous folding structures in one array are also possible.[87,90] This situation would be more relevant in vivo, where the linker length is not a constant. All in all, the structure of a folded nucleosome array in vivo can be heterogeneous with its local structures determined by the local parameters. The overall and local structures of nucleosome array compaction are the critical determinants of chromatin structural flexibility and gene accessibility.

Histone H4 has been implicated in internucleosomal interactions and compaction.[93] In particular, it has been reported that the basic residues on its N-terminal tail interact with the acidic patch of the H2A−H2B surface in a neighboring nucleosome.[4] Consequently, acetylation of these basic residues would weaken internucleosomal compaction and elevate gene accessibility, facilitating transcription and its activation. Acetylations at H4K12 and H4K16 have been coupled to transcription activation and active transcription.[94−96]

There are some epigenetic modifications that add a bulky group to the nucleosome. One example is SUMOylation.[18,97] SUMO has some binding partners, although the dissociation

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constant is relatively large at a micromolar level.[98] Upon binding, these proteins perform the cascading biochemical reactions that SUMO triggers. Another aspect of SUMOylation is that it adds a bulky chemical structure to the nucleosome that may interfere with the interactions between nucleosomes and inhibit compaction. Histones H4 K12 and K16 are targets of SUMOylation. As H4 tails are important for internucleosomal interactions, both acetylation and SUMOylation of these residues will have a significant impact on internucleosomal compaction.

To test this hypothesis, we developed a unique smFRET system with which we can observe how frequently nucleosomes bind one another and for how long a dinucleosome stack survives (Figure 8A).[99] The system is unique, because no previous method could probe the interactions between two identical nucleosomes at a single-molecule level in a time-resolved manner. From these measurements, we can determine the thermodynamic and kinetic stabilities of a dinucleosome stack and the effects of H4 acetylation and SUMOylation on these stabilities. Our system is devoid of any internucleosomal linker, consequently enabling measurements of the frequency of dinucleosome formation governed mostly by the entropy change. Therefore, this frequency should not be a function of histone modifications, unless long-range internucleosomal interactions exist. According to this assumption, the modification effect must be only on the lifetime of a dinucleosome stack. Our results indicate that H4 acetylations at K12 and K16 and SUMOylation at K12 indeed shorten the lifetime of a dinucleosome stack. Contrary to our expectation, the frequency of dinucleosome stack formation is also altered by these modifications, strongly suggesting that long-range internucleosomal interactions exist and that they are affected by these modifications. This is reasonable considering that the modifications are at the unstructured long tail of histone H4 that can reach out to another nucleosome at a distance farther than the Förster radius of the FRET pair.

These measurements resulted in the thermodynamic stabilities of the dinucleosome stacks with H4K12 SUMOylation and H4K16 acetylation that turned out to be lower than that of the wild-type dinucleosome by more than k_BT at 25 °C (Figure 8D). The results indicate that spontaneous internucleosomal compaction into a higher-order structure can be modulated by these modifications, confirming the long-hypothesized mechanism of histone tails regulating chromatin compaction via epigenetic modifications. Further investigations with proteins participating in chromatin compaction such as linker histone H1[91,92] will elucidate how much the thermodynamics and kinetics of nucleosome array compaction are governed by the spontaneous internucleosomal interactions and how much could be due to other factors in vivo.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

Many epigenetic modifications are small and ubiquitous, suggesting that their biophysical contributions to gene regulation can be significant. In particular, they often play direct roles in regulating the thermodynamic and kinetic stabilities of nucleosomes and nucleosome stacks and, subsequently, DNA-histone and nucleosome-nucleosome interactions. These roles are highly significant points of investigation, as the accurate regulation of the intra- and internucleosomal interactions between histone and DNA are critical to maintaining the healthy life and proliferation of a cell. More direct and accurate evaluation of these effects on gene regulation would entail investigations on the dynamics of various DNA-templated processes such as transcription and chromatin remodeling. Because of the complexity of the systems and their dynamics, single-molecule approaches will continue to make significant contributions to their investigations.

AUTHOR INFORMATION

Corresponding Author

*E-mail: txl18@psu.edu.

ORCID

Tae-Hee Lee: 0000-0003-2034-6394

Notes

The author declares no competing financial interest.

Biography

Tae-Hee Lee received his Ph.D. in physical chemistry from Georgia Institute of Technology (Atlanta, GA) in 2004 followed by his postdoctoral training in single-molecule biophysics at Stanford University (Stanford, CA). He joined the Chemistry department at the Pennsylvania State University in 2007 as an Assistant Professor and became a Professor in 2019. His current research focuses on gene regulation mechanisms in eukaryotes based on single-molecule fluorescence spectroscopy and microscopy. One of the goals is to elucidate the biophysical roles of chemical modifications on the nucleosome in gene regulation.

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