DISSERTATION

THE EFFECTS OF THE HISTONE CHAPERONE AND HISTONE MODIFICATIONS ON NUCLEOSOME STRUCTURE

Submitted by

Tao Wang

Department of Biochemistry and Molecular Biology

In partial fulfillment of the requirements

For the Degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Summer 2016

Doctoral Committee:

Advisor: Karolin Luger

Laurie Stargell
Tingting Yao
Robert M. Williams

ABSTRACT

THE EFFECTS OF THE HISTONE CHAPERONE AND HISTONE MODIFICATIONS ON NUCLEOSOME STRUCTURE

The nucleosome, composed of 147-bp DNA and a histone octamer, is the basic unit of chromatin in eukaryotes, which is considered as a barrier for all DNA dependent processes. Understanding how nucleosome structure is regulated provides new insights into pivotal cellular processes. Histone modifications and histone chaperones have potential roles in the regulation of nucleosome structure.

Here, I investigated the role of FACT in regulating nucleosome structure. FACT (FAcilitate Chromatin Transcription) is a conserved histone chaperone that is essential for gene transcription elongation. Our biochemical data show that FACT is not only a H2A-H2B chaperone, but also a H3-H4 chaperone. By binding H3-H4, FACT facilitates tetrasome assembly. In the presence of H2A-H2B, FACT facilitates H2A-H2B deposition onto tetrasomes and hexasomes, and thus promotes nucleosome assembly. FACT is also able to tether partial nucleosome components, composed of a histone hexamer and DNA, and results in forming an unstable complex. Interaction with H2A-H2B is essential for FACT binding to tetrasomal H3-H4. In order to hold a histone hexamer, FACT also stabilizes dimer-tetramer interaction. Previous study shows that H2BK120ub facilitates FACT function in gene transcription with the help of other transcription factors. Here, we show that H2AK119ub and H2BK120ub have no effects on FACT-H2A-H2B interaction and FACT assembly activity.

The role of select histone modifications in nucleosome structure was also determined in this dissertation. Histone modifications selected in this work are located at the entry-exit region of nucleosomal DNA. By using biochemical approaches, we find that H3Y41E (mimic phosphorylation) and H3R45E (mimic phosphorylation) affect the shape of nucleosome by facilitating nucleosomal 'DNA breathing'.

Tao Wang
Department of Biochemistry and Molecular Biology
Colorado State University
Fort Collins, CO, 80523
Summer 2016

ACKNOWLEDGEMENTS

It is my honor to pursue the PhD at Colorado State University in Fort Collins. In the department of Biochemistry and Molecular Biology, I met a lot excellent scientists. Everyone in the department is very helpful and collaborative. I enjoyed the working time in this department.

I feel really happy and lucky to join Dr. Karolin Luger's lab. I wish to express my gratitude to Dr. Karolin Luger. She is a brilliant mentor, and enlightens me how to be a scientist. She also gave me lots of valuable advice to design the experiments, interpret the results and keep the motivation about the research. More importantly, she shares her experience with me, and guides me to build my career path. During my six-year graduate study, I cannot express how much I learned from her. I believe that the experience I learned from her will benefit me for my life time.

Additionally, I would like to thank my committee members, Dr. Laurie Stargell, Dr. Tingting Yao, and Dr. Robert M. Williams for their valuable suggestions and help. Their support is very important for me.

I also appreciate the help from the current or past members from Dr. Karolin Luger's lab. Dr. Karolin Luger's lab is like a big family. Everyone in her lab is extremely helpful and friendly. Specially, I want to thank Dr. Mark van der Woerd, Dr. Serge Bergeron and Daniel Krzizike. Dr. Mark van der Woerd helps me collect SAXS data, and also shows me how to analyze SAXS data. I appreciate the advice and help from Dr. Serge Bergeron. The assay he developed is extremely important for my project. Daniel Krzizike helps me

a lot in analytical ultracentrifugation experiment. I am really grateful for his expertise and time.

Eventually, I would like to thank my family members. My mother and father always support me. Without their love, I would not go through my exciting PhD journey.

TABLE OF CONTENTS

CHAPTER 1

REVIEW OF LITERATURE

1.1 Nucleosome structure

DNA is the genetic material that is essential for life. Over two meters of DNA are organized by tightly wrapping around a histone octamer to fit into the tiny cell nucleus. Histone octamer consists of two copies of H2A-H2B heterodimers and one copy of a H3-H4 heterotetramer. Each histone contains a basic N-terminal tail, and a structured domain. H2A also has a C-terminal tail (Figure 1.1) (Luger et al. 1997). The nucleosome, which is comprised of 146-bp DNA and the histone octamer, is the basic unit of chromatin in eukaryotes. In the presence of the linker histone H1, nucleosomes are further compacted to a 30 nm fiber (Allan et al. 1980, Robinson and Rhodes 2006), which is considered as the secondary structure of chromatin. However, it was claimed that no 30 nm fiber was observed in human mitotic chromosomes (Nishino et al. 2012). Thus, the existence is still controversial. The higher-order chromatin structure is formed by oligomerization of nucleosome arrays (Figure 1.2) (Li and Reinberg 2011). Each nucleosome presents an obstacle for various DNA dependent processes, such as gene transcription, DNA replication and DNA damage repair (Fischle et al. 2003, Kulaeva et al. 2007, Leman and Noguchi 2013, Price and D'Andrea 2013).

Nucleosome stability is very dynamic. Intermediate states of nucleosomes were described over last decades (Böhm et al. 2011, Floer et al. 2010, Poirier et al. 2008, Zhang et al. 2011). For example, DNA can highly frequently be unwrapped from histone

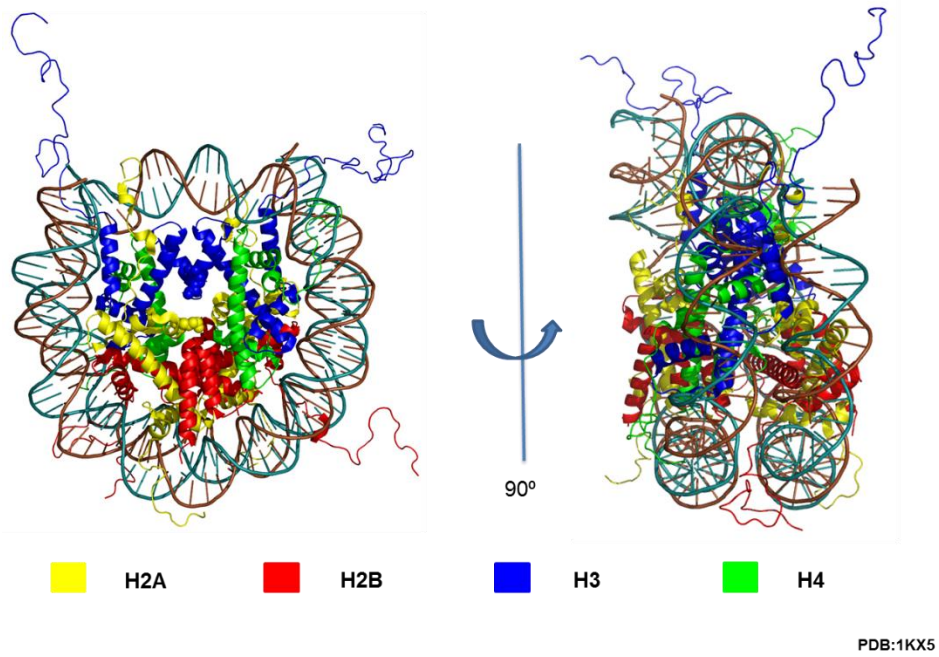


Figure 1.1 Crystal structure of nucleosome. 146-bp DNA wraps around a histone octamer, and forming a nucleosome. The histone octamer is comprised of two copies of each histone, which are indicates in different colors. Left panel is front view of nucleosome, while right panel is side view of nucleosome.

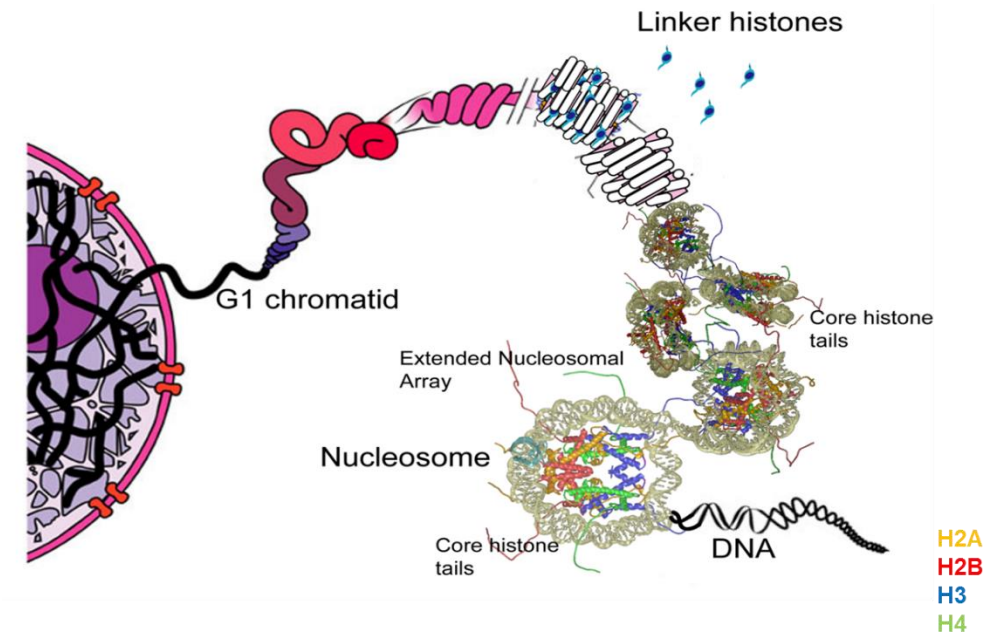


Figure 1.2 Higher order chromatin structure. Nucleosome is considered as the basic unit of chromatin in eukaryotes. With help of the linker histone H1, nucleosomes are further compacted to a 30 nm fiber, which is the secondary structure of chromatin. However, it was claimed that no 30 nm fiber was observed in human mitotic chromosomes. Thus, the existence is still controversial. The higher-order chromatin structure is formed by oligomerization of nucleosome arrays (Luger and Hansen 2005; Nishino et al. 2012).

octamers in a process termed ‘DNA breathing’ (Poirier et al. 2008). Besides, ‘open state’ nucleosomes were determined by single molecule fluorescence resonance energy transfer (FRET) (Böhm et al. 2011). It was discovered that H2A-H2B heterodimer could disassociate from the H3-H4 heterotetramer before being released from nucleosomes. Different forms of nucleosomes are in thermal equilibrium under physiologic conditions. Several factors have potential effects on nucleosome stability, such as histone posttranslational modifications (PTMs), histone chaperones, ATP-dependent chromatin remodelers and histone variants (Andrews et al. 2010, Goldman et al. 2010, Howman 2000, Stoler et al. 1995).

1.2 The effects of Histone Posttranslational Modifications (PTMs) on nucleosome structure

Histone PTMs have been described in the last century (Phillips 1963). Increasing numbers of histone PTMs has been identified, such as acetylation, methylation, phosphorylation and ubiquitination. It was suggested that histone PTMs affect chromatin structure by direct or indirect mechanisms (Cosgrove et al. 2004). Decades ago, most histone PTMs were identified at each core histone tail. It was proposed that serving as a binding site for regulatory proteins is the major potential roles for histone PTMs from one or more histone tails, and this concept was termed ‘histone code’ (Strahl and Allis 2000). For example, Heterochromatin Protein 1 (HP1) containing chromodomain binds to H3K9me3, which results in transcription repression (Bannister et al. 2001, Jacobs et al. 2001). Alternatively, histone PTMs may directly regulate chromatin structure. Indeed, histone modifications may weaken the interactions between DNA and histone, histone and histone, or histone and DNA-histone complexes (Brehove et al. 2015, Jørgensen et

al. 2013, Ramachandran et al. 2011). These histone modification sites likely locate at the buried regions of nucleosomes. No much data shows histone PTMs at each core histone tail dramatically affect nucleosome stability in a direct way. Most of histone PTMs selected in this project are occurred at histone structural domain.

Acetylation of histones was described in 1963 (Phillips 1963). Acetylation of histones is the introduction of an acetyl group to lysine residues (K), which is achieved by enzymes containing “HAT” domain (histone acetyltransferase). Acetylation of histones neutralizes the positive charges from the side chain of lysine residues and weakens the charge dependent binding of histones to DNA, or adjacent histones. Decades ago, Bode and his colleagues suggested that hyperacetylated histones increase nucleosomal DNA accessibility, which facilitates protein binding to target DNA (Bode et al. 1983, Bode et al. 1980). Also, it was found that hyperacetylated histones change the shape of nucleosomes (Bauer et al. 1994). However, at that time it was not determined which modification sites are responsible for this phenomenon, due to the difficulty of synthesizing site-specific acetylated lysine at structural regions of each core histone. Until 2009, the approach to synthesize acetylated histone was developed by Dr. Chin and colleagues (Neumann et al. 2009). By their approach, acetyl-tRNA synthesase (AckRS)/$tRNA_{CUA}$ pair in response to an amber codon. H3K56ac, located at the entry-exit region of nucleosomes, was synthesized by this approach. It has been shown that H3K56ac facilitates nucleosomal DNA breathing (Neumann et al. 2009). Additionally, nucleosomes with H3K56Q mutants (mimic acetylated H3K56) are more sensitive to Micrococcal nuclease (MNase) than nucleosomes with WT octamers (Masumoto et al. 2005), which indicates more ‘open’ chromatin structure. All these experimental results were consistent with the hypothesis

that histone PTMs throughout the entry and exit regions of nucleosomes facilitate DNA unwrapping (Koopmans et al. 2007). Besides, Andrew and his colleagues found that H3K56ac also makes the formation of tetrasome less favorable (Andrews et al. 2010). The binding affinity of tetramer with H3K56ac to DNA is ~15 fold weaker compared to tetramer with unmodified canonical histones (Andrews et al. 2010). Thus, H3K56ac affects nucleosome stability by either facilitating DNA unwrapping or preventing tetrasome formation.

Acetylation of H3K122 is another intensively studied modification site, which was discovered in 2003 (Zhang et al. 2003). This residue locates at dyad axis of the nucleosomes. Manohar and co-workers claimed that H3K122ac does not increase the DNA accessibility by restriction enzyme kinetic studies (Manohar et al. 2009). Recently, H3K122ac has been identified in human cells by mass spectrometry experiments (Tropberger et al. 2013). It has been shown that H3K122ac stimulates gene transcription in vitro by directly affecting nucleosomes structure (Molina-Serrano and Kirmizis 2013). Indeed, H3K122ac promotes nucleosome disassembly by disrupting histone-DNA interaction (Manohar et al. 2009, Simon et al. 2011).

Methylation of histones is the introduction of methyl group to lysine or arginine residues, which is mediated by methyltransferase. Mono- (me), di- (me2) and tri-methylation (me3) of lysine have been identified in vivo (Shepherd et al. 1971). For different degrees of methylation, different biological functions have been reported (Lee et al. 2005, Simon et al. 2007). However, no evidence exists to show that different degrees of methylation directly affect chromatin structure by distinct mechanisms.

Methylation of histones affects chromatin structure by different mechanisms, compared to histone acetylation. Although histone methylation conserves the positive charge of lysine or arginine, it was suggested that histone methylation facilitates nucleosome disassembly by arrangement of side chains of amino acids, which alters the surface of nucleosomes (Zhou et al. 2007). For example, H3K42 locates at histone core domain, which is involved in DNA-histone interactions. Recently, it was discovered that H3R42me2 stimulates gene transcription in vitro transcription system (Casadio et al. 2013). Although its mechanism still needs to be elucidated, it was speculated that H3R42me2 weakens the interactions between DNA and the histone octamer.

Additionally, histone methylation has direct effects on higher-order chromatin structure. For example, trimethylation at H4K20 results in the enhancement of chromatin condensation (Lu et al. 2008). It was suggested that histone methylation may severe as a barrier for histone acetylation, which could weaken histone octamers interaction with DNA (Cosgrove et al. 2004). In case of H3K64me3, H3K64 lies within histone structural region and interacts with DNA. Tri-methylation of this residue is enriched in heterochromatin (Daujat et al. 2009). This modification is a repressive mark for gene transcription (Daujat et al. 2009). However, it is still unknown how H3K64me3 regulates chromatin structure. Likely, methylation of H3K64 just simply prevents H3K64ac. Moreover, the half-life of histone methylation is proximally equal to the half-live of histone itself (Byvoet and Shepherd 1972). Thus, histone methylation may serve as a stable obstacle for histone acetylation. In sum, the role of histone methylation in chromatin structure is more complicated than that of histone acetylation.

Phosphorylation of histones is the introduction of a phosphate group to the side chain of serine (S), threonine (T) or tyrosine (Y) residues, which is achieved by kinases. The phosphate group imparts a negative charge at side chain of modified residue, which may disturb DNA-histone interaction. Thus, it was suggested that histone phosphorylation affects chromatin structure by the similar mechanisms as histone acetylation (Banerjee and Chakravarti 2011). Mirsky and co-works found that phosphorylated histone has less effect on blocking DNA accessibility than unphosphorylated one (Mirsky et al. 1972). This result indicates that histone phosphorylation may have potential effects on chromatin structure. H3Y41 phosphorylation was identified in human leukemic cells, which is modified by Janus kinase 2 (JAK2) (Dawson et al. 2009). H3Y41 is positioned at the N-terminal tail of H3. The side chain of H3Y41 is located in a minor groove of DNA, which interacts with DNA. It was reported that this modification is associated with active promoters (Dawson et al. 2012). The role of this modification at active promoters needs to be elucidated. However, mutation of H3Y41 facilitates nucleosomal DNA unwrapping (Ferreira et al. 2007), which indicates that phosphorylation of H3Y41 may have similar effects on chromatin structure as H3K56ac.

Ubiquitination (ub) of histones is the introduction of a ubiquitin molecule to the side chain of lysine residues, which is achieved by ubiquitin ligase (E3) in the presence of ubiquitin activating enzyme (E1) and ubiquitin conjugating enzyme (E2) (Scheffner et al. 1995). To date, several histone ubiquitination sites were identified. most of them are located at the solvent-accessible surface of nucleosomes such as H2AK119 and H2BK123 in yeast (H2BK120 in vertebrate) (Goldknopf et al. 1975, Shilatifard 2006, Thorne et al. 1987, Zhang 2003). The ubiquitination of H2A was discovered in 1975, while H2Bub was

reported in 1980 (Goldknopf et al. 1975, West and Bonner 1980). In vitro chromatin immunoprecipitation (ChIP) data suggest that H2Aub is mainly related to satellite repeats, while H2Bub is associated with active gene transcription (Minsky et al. 2008, Zhu et al. 2011). It indicates that H2Aub and H2Bub has different biological functions.

The first discovered E3 ubiquitin ligase related to H2AK119ub is RING1B, which is a polycomb group protein (Wang et al. 2004). Subsequently, several E3 ubiquitin ligases responsible for H2Aub have been identified, such as breast cancer type 1 and 2A-HUB/hRUL138 (Chen et al. 2002, Zhou et al. 2008). Similarly, H2B-specific E3 ubiquitin ligases have been discovered and characterized, such as Rad6 and RNF20 (Robzyk et al. 2000). Besides, histone ubiquitination is a reversible reaction. H2A-specific deubiquitination enzymes have also been identified, such as USP16, 2A-DUB and BAP1 (Joo et al. 2007, Nakagawa et al. 2008, Scheuermann et al. 2010). In contrast, Ubp8 and Ubp10 are associated with H2B deubiquitination (Emre et al. 2005, Henry et al. 2003). Ubp8 is a component of SAGA. Crystal structure of SAGE DUB module (Ubp8/Sgf11/Sus1/Sgf73) with ubiquitinated nucleosome shows that DUB module mainly interacts with H2A-H2B dimer. The catalytic domain in Ubp8 makes additional interactions with H2B (Morgan et al. 2016).

Compared to the above mentioned histone modifications, ubiquitin is a relatively large molecule, whose molecular weight is about 8.5 KDa (Goldstein et al. 1975). Thus, any lysine residue in the solvent-inaccessible regions of nucleosomes is ubiquitinated, which may lead to disrupt nucleosome structure. However, no experimental data exists to show that histone ubiquitinations directly have an impact on nucleosome structure. Likely, these modification sites only serve as a binding site for other regulatory proteins. Nevertheless,

two histone ubiquitinations have potential roles in regulating chromatin structure (Batta et al. 2011, Ikura et al. 2007). In yeast, H2BK123ub plays a role in nucleosome assembly. Loss of this modification by deletion of Rad6 decreases the occupancies of nucleosomes in genome-wide (Batta et al. 2011). H2AX is a histone H2A variant. It was suggested that ubiquitination of H2AXK119 may promote nucleosome disassembly by decreasing the binding affinity of modified histones to DNA (Ikura et al. 2007). Another novel ubiquitination site (H2BK34) is located at H2B N-terminal tail (Tweedie-Cullen et al. 2009). It was suggested that H2BK34ub may directly affect the histone-DNA interactions, and results in destabilizing nucleosome (Wu et al. 2011). Again, the precise role of these three modified histones in chromatin structure still needs to be elucidated.

The difficulty to prepare an analogue of histone ubiquitination is the main obstacle to establish the effects of histone ubiquitination on chromatin structure. Recently, H2BK34 with ubiquitinated analogue was successfully prepared by chemical-ligation method (Siman et al. 2013). In addition, site-specific ubiquitinated histone mimics were also synthesized by a straightforward crosslinking method (Long et al. 2014). H2AK119ub and H2BK120ub were successfully synthesized by this approach.

1.3 The role of histone chaperone in regulating nucleosome structure

Originally, histone chaperone was discovered as a family of protein with acidic domain. The main function of histone chaperones, proposed soon after their discovery, is to prevent DNA-histone aggregation (Laskey et al. 1978). To date, histone chaperone refers to a family of protein involved in nucleosome assembly or disassembly (Burgess and Zhang 2013). Distinct histone chaperones have preference to bind either H2A-H2B or H3-

H4. For example, Asf1 (Anti-Silencing Function protein 1) is a H3-H4 histone chaperone. It binds H3-H4 dimer with 2.5 nM binding affinity (Donham et al. 2011). CAF1 (Chromatin Assembly Factor 1) is an another compelling example. It binds to $(H3\text{-}H4)_2$ tetramer with similar affinity as Asf1 (Winkler et al. 2012). In contrast, several histone chaperones bind both H3-H4 and H2A-H2B. For instance, Nap1 (Nucleosome Assembly Protein 1) binds both H3-H4 and H2A-H2B with similar affinity (Andrews et al. 2008). Similarly, FACT (FAcilitates Chromatin Transcription) also binds both H2A-H2B and H3-H4 (Kemble et al. 2015, Kemble et al. 2013, Winkler et al. 2011). Mechanically, it is still not clear how some histone chaperones prefer to binding H2A-H2B or H3-H4.

The nucleosome is assembled through multiple steps. The old idea was that nucleosome assembly is started from one H3-H4 heterotetramer deposition onto DNA forming tetrasome, and followed by two H2A-H2B heterodimers binding to tetrasome to form a nucleosome (Figure 1.3) (Smith and Stillman 1991). It was suggested that H3-H4 heterotetramer was formed through H3-H3' four helix-bundle (Arents and Moudrianakis 1995). They may separate into two H3-H4 heterodimers in the absence of DNA (Banks and Gloss 2004). Thus, nucleosomes assembly may be initiated by the deposition of one H3-H4 heterodimer onto DNA, followed by deposition of a second H3-H4 heterodimer deposition to form tetrasome, finished by two H2A-H2B heterodimers binding to tetrasome. Nucleosome disassembly may happen through the reverse pathway. Histone chaperones play an important role in each step of nucleosome assembly/disassembly.

Firstly, nucleosome assembly starts from tetrasome assembly. Several histone chaperones are involved in this process, such as Asf1, CAF1 and Rtt109 (Donham et al. 2011, Driscoll et al. 2007, Kadyrova et al. 2013). The crystal structure of Asf1-(H3-H4)

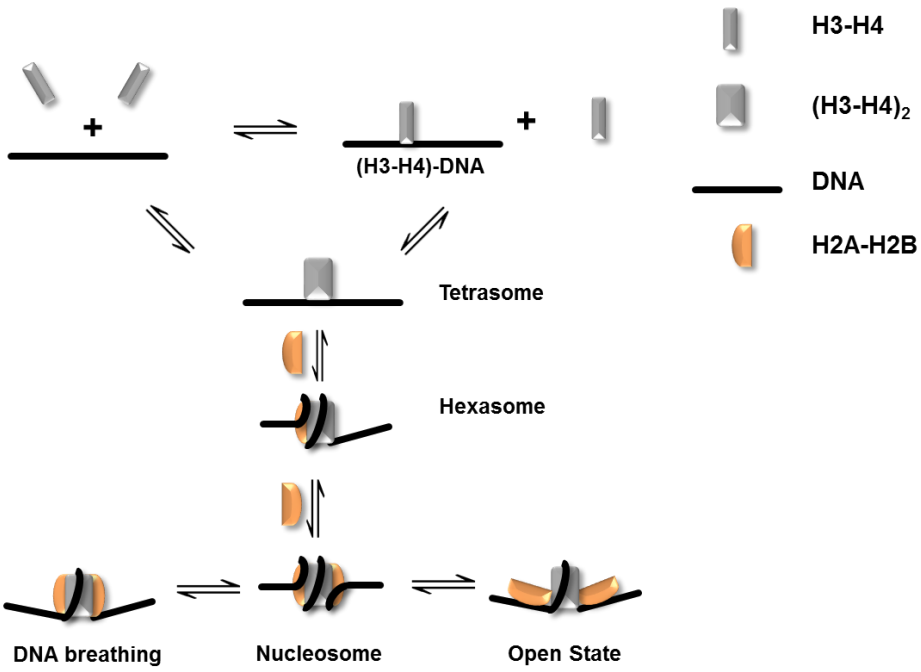


Figure 1.3 Nucleosomes assemble and disassemble through multiple-steps. Nucleosome assembly is initiated with the deposition of one (H3-H4)2 heterotetramer onto DNA forming a tetrasome, followed by two H2A-H2B heterodimers binding to tetrasome to form a nucleosome. Intermediate states of nucleosome have been uncovered, such as DNA breathing and 'open state'. Nucleosome disassembly could be accomplished in a reverse way (Polach and Widom 1995; Böhm et al. 2011).

shows that Asf1 binds a H3-H4 dimer through disrupting H3-H3' interaction (English et al. 2006). It has been reported that Asf1 facilitates H3-H4 dimer deposition onto DNA via preventing nonspecific histone-DNA interaction (Andrews et al. 2008). Additionally, Asf1 delivers H3-H4 dimer to Rtt109, and results in H3K56ac by Rtt109 (Driscoll et al. 2007). Then, tetrasome is assembled with the help of CAF1. It was suggested that CAF1 is involved in DNA replication-dependent nucleosome assembly (Stillman 1986, Stillman and Gluzman 1985). Next, nucleosome assembly process is finished by the deposition of H2A-H2B onto tetrasome and hexasome. Although several H2A-H2B chaperone has been discovered, most of them is involved in H2A-H2B displacement. For example, Nap1 is a H2A-H2B chaperone (Andrews et al. 2008). However, Nap1 does not facilitate H2A-H2B deposition onto tetrasome or hexasome. Instead, biochemical studies show that Nap1 disassembles nucleosome by removing H2A-H2B from nucleosome (Levchenko and Jackson 2004). Thus, it is still not clear how H2A-H2B deposits onto tetrasome during gene transcription and DNA replication.

1.4 FAcilitate Chromatin Transcription (FACT)

1.4.1 FACT is a conserved histone chaperone.

FACT is a heterodimer of Spt16 (suppressor of Ty 16) and SSRP1 (structure-specific recognition protein 1) in higher eukaryotes (Figure 1.4) (Orphanides et al. 1998). In yeast, the SSRP1 subunit is separated into two subunits, Pob3 (Pol1-binding protein 3) and Nph6 (HGM family protein) (Singer and Johnston 2004). Biophysical studies suggest that FACT binds both H2A-H2B and H3-H4, which suggests FACT is not only a H2A-H2B chaperon, but also a H3-H4 chaperone (Kemble et al. 2015, Tsunaka et al. 2016).

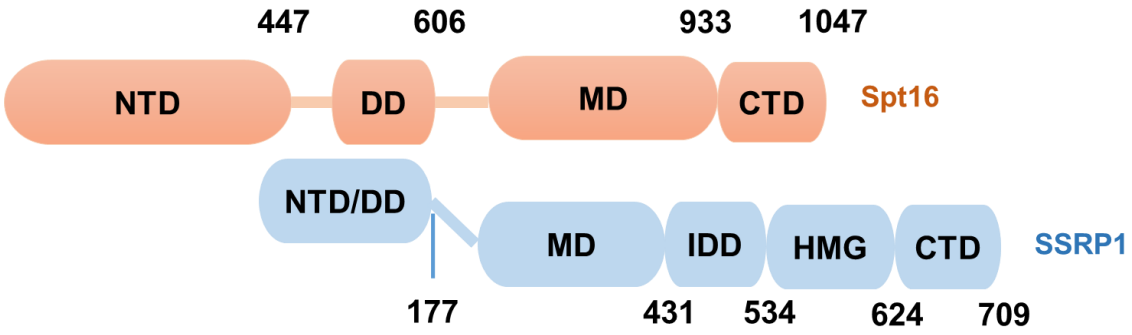


Figure 1.4 Structural alignment of hFACT. hFACT, containing multiple functional domains, is comprised of two subunits (Spt16-SSRP1). Spt16-NTD is an 'aminopeptidase-like' domain. Spt16MD is involved in the interaction with H3-H4 tetramer. Recently, Spt16MD-AID-H3-H4 crystal structure has been solved. Controversially, Spt16MD-H2A-H2B crystal structure has also been solved, which indicates that Spt16MD interacts with H2A-H2B. The reason for these contradictions is that Spt16MD-H2A-H2B crystal structure was solved by using a translational fusion of Spt16MD with H2A-H2B, which may cause nonspecific interaction. In agreement, crystal structure of Spt16CTD-H2A-H2B suggests that Spt16CTD interacts with H2A-H2B dimer, which is essential for FACT activity. Additionally, SSRP1 contains a HMG domain, which has DNA binding property (Kemble et al. 2015; Yang et al. 2016; Hondele et al. 2013; Winkler and Luger 2011; Tsunaka et al. 2016).

It is still unknown if FACT have preference to binding H2A-H2B or H3-H4. The consequence of these physical interactions are also unclear.

1.4.2 FACT contains multiple function domains.

From a structural viewpoint, FACT contains multiple function domains. Structural study of the Spt16 N-terminal domain (NTD) reveals an aminopeptidase-like domain, which is composed of a N-terminal lobe and a C-terminal lobe (Figure 1.5) (Marciano and Huang 2016). It has been reported that the yeast and human Spt16 NTD bind H3-H4 with low micromolar affinity (Hondele et al. 2013, Kemble et al. 2013). However, hSpt16 middle domain (Jessulat et al.) also interacts with H3-H4. A recent crystal structure of the Spt16 MD-(H3-H4) complex reveals that Spt16 MD binds a $(H3\text{-}H4)_2$ tetramer at two separate sites (Figure 1.6) (Tsunaka et al. 2016). One binding site involves the H4 L1 loop, which is bound by DNA in the context of the nucleosome. The other binding site is located at the H2A docking site at the surface of H3 (α3 helix)-H3' (α2 helix)-H4' (α1 helix). Spt16 C-terminal domain (CTD) interacts with H2A-H2B. Yeast Spt16 CTD-(H2A-H2B) crystal structure shows that Spt16 CTD mainly binds H2B, and weakly interacts with H2A (Figure 1.7) (Kemble et al. 2015). Similarly, it has been shown that hFACT ΔCTD (Spt16 ΔCTD) impedes FACT-(H2A-H2B) interaction (Belotserkovskaya et al. 2003, Winkler et al. 2011). Additionally, biochemical studies suggest that interaction of H2A-H2B does not preclude FACT-(H3-H4) interaction (Kemble et al. 2015). Thus, it was speculated that FACT may bind H2A-H2B and H3-H4 simultaneously. Since hFACT binds one H2A-H2B dimer, it has been suggested that hFACT binds to a histone hexamer, composed of one $(H3\text{-}H4)_2$ tetramer and one H2A-H2B dimer. Accordingly, a model has been proposed that yFACT

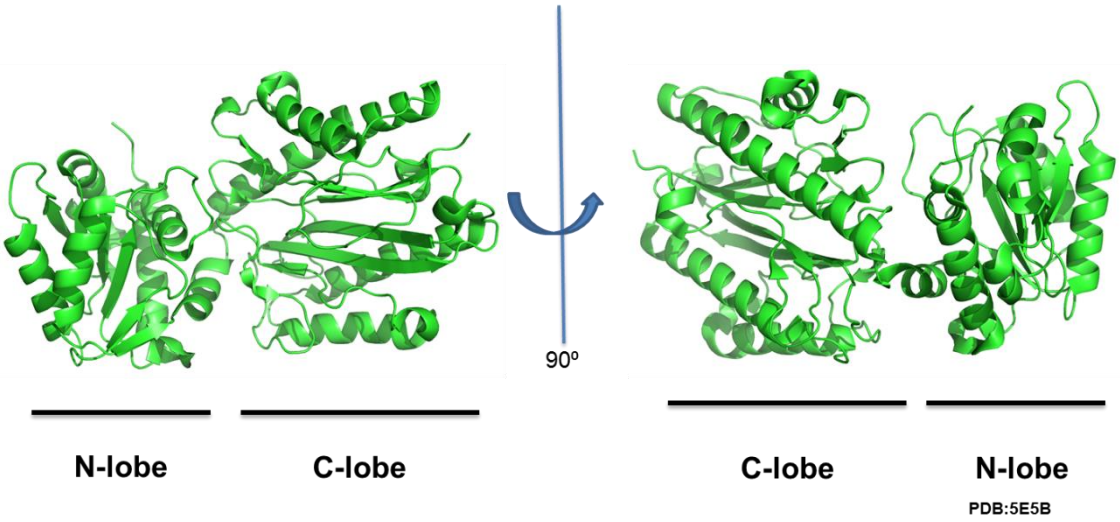


Figure 1.5 Crystal structure of Spt16-NTD. Structural study of the human Spt16 N-terminal domain (NTD) reveals an aminopeptidase-like domain. In addition, Spt16-NTD is composed of a N-terminal lobe and a C-terminal lobe (Marciano and Huang 2016).

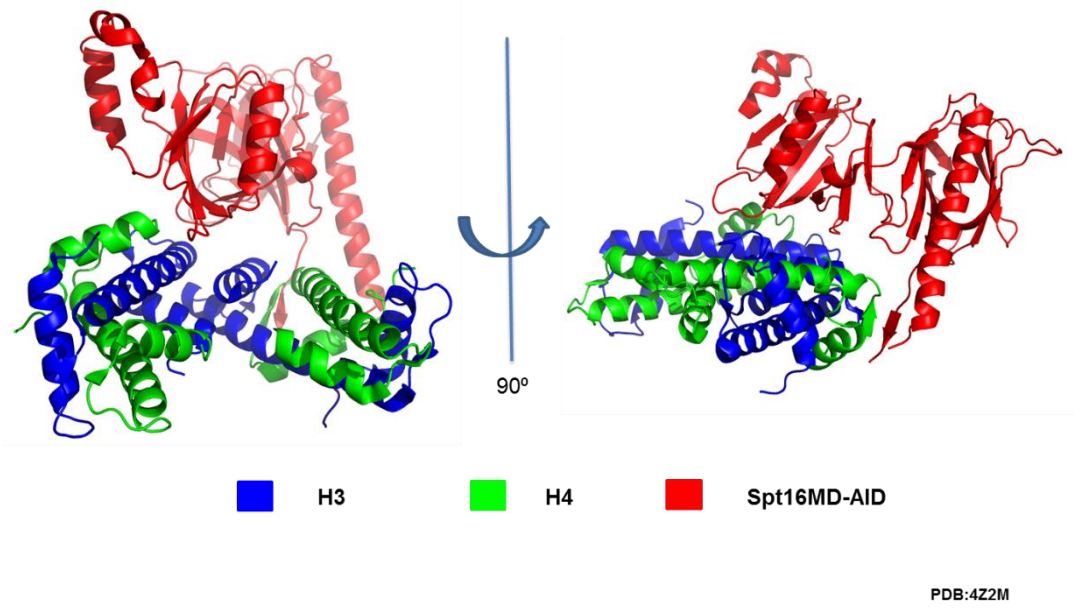


Figure 1.6 Crystal structure of Spt16MD-AID-(H3-H4). Spt16MD-AID was co-crystallized with $(H3\text{-}H4)_2$ tetramer. From structural view, Spt16-MD interacts with $(H3\text{-}H4)_2$ tetramer at two separate sites. One binding site is located in the H4 L1 loop, the other one is located at the surface of H3-H3'-H4', which interacts with H2A-H2B in the context of a nucleosome (Tsunaka et al. 2016).

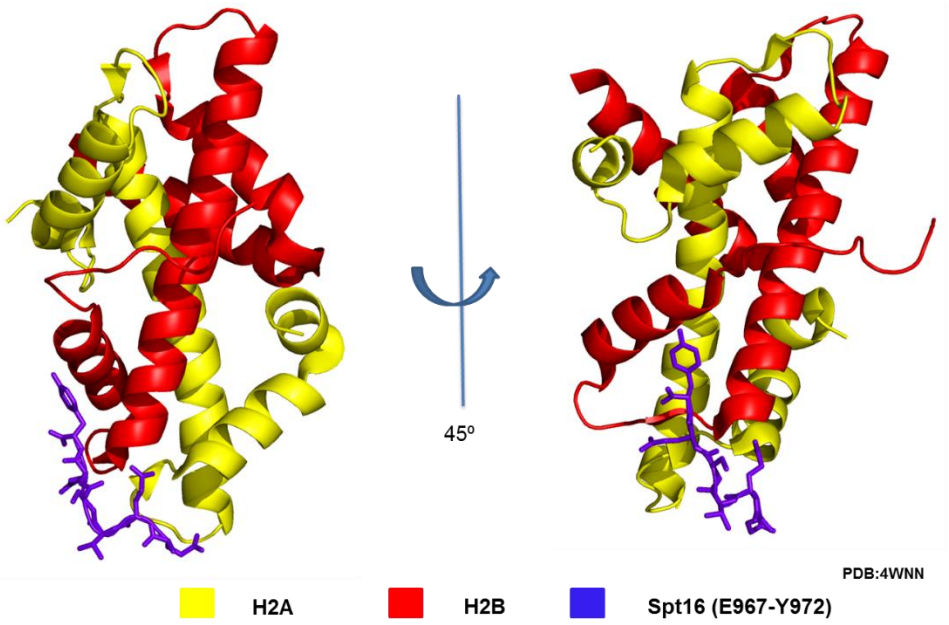


Figure 1.7 Crystal structure of Spt16-CTD-(H2A-H2B). Spt16 (E967-Y972) peptide was co-crystallized with H2A-H2B dimer. From structural view, Spt16-CTD mainly interacts with H2B, with minor contacts with H2A (Kemble et al. 2015).

may hold the components of nucleosome during gene transcription (Kemble et al. 2015). As RNA pol II goes through, FACT rapidly reassembles nucleosome.

Human SSRP1 contains a DNA binding domain, the HMG domain. An intrinsically disordered domain (IDD) is located at the upstream of HMG domain. It has been shown that phosphorylation in the IDD causes IDD binding to HMG, resulting in disrupting FACT-DNA interaction (Tsunaka et al. 2009). Dephosphorylated FACT binds nucleosomal DNA nonspecifically (Tsunaka et al. 2009). In order to prevent FACT-DNA nonspecific interaction, phosphorylated FACT was utilized in most biochemical studies (Tsunaka et al. 2016, Winkler et al. 2011).

1.4.3 The role of FACT in regulating nucleosome structure

FACT was discovered as a transcription elongation factor (Orphanides et al. 1998). Biochemical studies suggest that FACT facilitates gene transcription by removing H2A-H2B from nucleosome (Belotserkovskaya et al. 2003). Indeed, structural studies reveal that FACT-(H2A-H2B) interaction is incompatible with nucleosomal DNA-(H2A-H2B) interaction (Kemble et al. 2015). It has been shown that FACT removes H2A-H2B from DNA (Winkler et al. 2011). Additionally, FACT-(H2A-H2B) interaction is essential for overcoming nucleosome barrier during gene transcription (Hsieh et al. 2013). All these evidence support the idea that FACT is a nucleosome disassembly factor.

However, genetic data suggest that FACT temperature-sensitive mutant decreases nucleosome occupancy (Kaplan et al. 2003). FACT also reassembles nucleosomes behind RNA polymerase II. It was suggested that FACT maintains nucleosome structure by redepositing H3-H4 (Jamai et al. 2009). Besides, incorporation of new H3 increases in

the absence of Spt16 (Voth et al. 2014), indicating that FACT facilitates tetrasome reassembly. In this way, FACT may maintain histone modification patterns by re-deposition of preexisting H3-H4. Similarly, FACT temperature-sensitive mutants adversely affect nucleosome deposition, as shown by an in vivo Chromatin Immunoprecipitation (ChIP) assay (Nguyen et al. 2013). These genetic studies support the idea that FACT is a nucleosome assembly factor. However, the mechanism by which it assembles nucleosomes needs to be elucidated.

Due to these contradicting data, the role of FACT in regulating nucleosome dynamics needs further investigation. Perhaps, FACT is able to both assemble and disassemble nucleosomes. According to genetic data, FACT may assemble nucleosomes by facilitating tetrasome assembly (Nguyen et al. 2013). FACT disassembles nucleosome by removing H2A-H2B. Several histone chaperones have both nucleosome assembly and nucleosome disassembly activity. For example, Nap1 assembles nucleosome in vitro (Fujii-Nakata et al. 1992). Nap 1 also enhances DNA accessibility by disassembling nucleosome (Levchenko and Jackson 2004). Similarly, Nap1 binds both H2A-H2B and H3-H4 as mentioned above. Assuming that FACT has both assembly and disassembly activity, the question how FACT switches its assembly/disassembly activity still needs to be addressed.

1.5 Linker Histones

Linker histone H1 (consisting of ~200 a.a.) is very abundant in the nucleus. Linker H1 histone is comprised of N-terminal tail, a globular domain and a highly basic C-terminal tail. It has been shown that H1 plays an important role in chromatin compaction (Thoma

et al. 1979). Biochemical study suggests that H1 histone binds to mononucleosome with picomolar affinity (White et al. 2016). Partial C-terminal tail (97 to 121 a.a.) is essential for H1 binding nucleosome linker DNA (Fang et al. 2011, White et al. 2016).

The question how H1 is removed from nucleosome still needs to be answered. Recently, it has been reported that histone PTMs at H3 N-terminal tail decrease H1 occupancy on nucleosome. Genetic data suggest that acetylation at H3 N-terminal tail precludes the occupancy of H1 (Stutzer et al. 2016). Additionally, biochemical study suggests that SET and hNap1 physically interact with H1, and results in linker histone eviction from chromatin (Zhang et al. 2015). Similarly, FACT also binds to H1 with ~35 nM affinity (Kalashnikova et al. 2013). The binding site of FACT at H1 is still not clear. Moreover, the role of FACT in H1-nucleosome interaction still needs to be elucidated.

1.6 Scope of this thesis

The work in this dissertation focuses on understanding how nucleosome structure is regulated. Firstly, FACT-histone binding affinity was determined by fluorescence-based plate assay. The stoichiometry of FACT-histones was measured by Analytical UltraCentrifugation (AUC). Additionally, we attempted to determine if FACT could bind H2A-H2B and H3-H4 simultaneously. Then, we asked how FACT regulates nucleosome structure. By using high resolution electrophoretic mobility shift assay (EMSA), and AUC, we determined that FACT does not disassemble properly folded nucleosomes.

Next, we investigated if FACT is a nucleosome assembly factor. An in vitro gel-based nucleosome assembly assay and MNase digestion assay were applied. We dissected which step of nucleosome assembly FACT is involved in. The role of FACT in gene

transcription was also investigated by using a defined in vitro transcription system. Fluorescence resonance energy transfer (FRET)-based competition plate assay was applied, in order to test if H2AK119ub or H2BK123ub disrupts FACT-(H2A-H2B) interaction. The contribution of mono-ubiquitination at either H2AK119 or H2BK120 in FACT assembly/disassembly activity was investigated. Additionally, the effects of histone ubiquitination on FACT activity during gene transcription were also determined.

We also determined how histone modifications regulate nucleosome structure. By using size exclusion chromatography with multiple angle light scattering, small angle X-ray scattering, restriction enzyme and MNase digestion assay, we were able to determine the effect of selected histone modifications on nucleosome structure. Several histone modifications were selected, due to their structural and biological importance. Since these selected histone modifications are mainly located in DNA entry-exit region of the nucleosome, we propose that these histone modifications facilitate DNA unwrapping from the surface of histone octamer.

CHAPTER 2

THE EFFECTS OF HISTONE CHAPERONE FACT ON NUCLEOSOME STRUCTURE

2.1 Summary

FACT was discovered as a transcription elongation factor, and plays an essential role in many chromatin-related activities. hFACT, composed of Spt16 and SSRP1, is a conserved histone chaperone. Our biochemical studies suggest that FACT is a nucleosome assembly factor that facilitates tetrasome assembly, as well as H2A-H2B deposition onto the tetrasome and hexasome. We also find that FACT tethers partial components of nucleosome via interaction with H2A-H2B and tetrasomal H3-H4, which results in forming a homogenous complex. Compare to nucleosome, DNA in this complex is less stably organized. FACT-H2A-H2B interaction and (H2A-H2B)-(H3-H4) interaction are crucial for the ability of FACT to bind to these nucleosome components. This work provides mechanistic insight into how FACT regulates nucleosome structure. All AUC-FDS experiments in this chapter were designed by me, and performed by Daniel Krzizike.

2.2 Introduction

The histone octamer consists of two copies of H2A-H2B heterodimers and one copy of a $(H3\text{-}H4)_2$ heterotetramer. 146-bp of DNA are wrapped 1.65 times around a histone octamer to form a nucleosome (Luger et al. 1997). Nucleosomes are considered to be barriers for DNA dependent processes, such as replication, transcription and DNA repair (Fischle et al. 2003, Kulaeva et al. 2007, Leman and Noguchi 2013, Price and D'Andrea

2013, Unnikrishnan et al. 2010). Therefore, the regulation mechanisms of nucleosome dynamics have received much attention during last decades.

Nucleosomes are assembled in multiple steps. It begins with the deposition of one H3-H4 heterotetramer onto DNA forming a tetrasome, followed by two H2A-H2B heterodimers binding to the tetrasome to form a nucleosome (Smith and Stillman 1991). Nucleosome disassembly is likely accomplished in the reverse pathway. Several factors, such as histone posttranslational modifications (PTMs), histone variants and histone chaperones have a potential role in the regulation of nucleosome dynamics (Andrews et al. 2010, Kadyrova et al. 2013, Venkatesh et al. 2012).

Histone chaperones are a family of proteins with an acidic domain, which bind to histones with high affinity. Histone chaperones are involved in nucleosome assembly or disassembly (Andrews et al. 2010, Eitoku et al. 2008, Huang et al. 2005, Kadyrova et al. 2013, Tyler et al. 1999). FACT (FAcilitates Chromatin Transcription) is a highly conserved histone chaperone in eukaryotes. Human (h) FACT is a heterodimeric complex, composed of Spt16 and SSRP1 (Orphanides et al. 1999). hFACT binds H2A-H2B with low nanomolar affinity (Winkler et al. 2011) and is thus widely considered as a H2A-H2B chaperone. The acidic region of Spt16 C-terminal domain (CTD) is essential for FACT-H2A-H2B interaction (Kemble et al. 2015). Besides, electrophoretic mobility shift assays (EMSA) suggest that yFACT binds to H2A-H2B and $(H3\text{-}H4)_2$ tetramer with similar affinity (~150 nM) (Kemble et al. 2015, Kemble et al. 2013). Several biochemical studies suggest that the Spt16 CTD is very important for FACT activity (Belotserkovskaya et al. 2003, Kemble et al. 2015, Tsunaka et al. 2016). In contrast, our previous work shows that hFACT binds to H3-H4 with ~700 nM affinity, and binds to H2A-H2B with ~30 nM affinity

by using fluorescence-based plate assay (Winkler et al. 2011). This discrepancy encouraged us to re-evaluate hFACT-H3-H4 binding affinity. The crystal structure of hSpt16 middle domain (MD)-acidic domain (AID)-$(H3\text{-}H4)_2$ shows that FACT-$(H3\text{-}H4)_2$ interaction is incompatible with (H2A-H2B)-(H3-H4) and H3-H4-DNA interaction in the context of nucleosome (Tsunaka et al. 2016). Together, this suggests that FACT is also an H3-H4 chaperone. Since H2A-H2B and H3-H4 bind distinct regions on FACT, it is possible that FACT tethers H2A-H2B and H3-H4 simultaneously. Indeed, it has been shown that hFACT binds a histone hexamer, composed of one H2A-H2B dimer and $(H3\text{-}H4)_2$ tetramer (Tsunaka et al. 2016).

FACT was discovered as an essential transcription enlongation factor (Orphanides et al. 1998). How FACT stimulates gene transcription and regulates nucleosome structure still needs to be addressed. Belotserkovskaya et al determined that FACT stimulates gene transcription by using an in vitro assembled chromatin template with general transcription factors. It was proposed that FACT displaces one or two H2A-H2B dimers from nucleosomes during transcription (Belotserkovskaya et al. 2003). Histone mutations, located in the interface between H2A-H2B dimer and $(H3\text{-}H4)_2$ tetramer interface, reduce the need for FACT to be fully active (McCullough et al. 2013). Taken together, this suggests that FACT is a nucleosome disassembly factor. However, yFACT also reassembles nucleosomes in the wake of RNA polymerase II by an in vivo ChIP assay (Jamai et al. 2009). Similarly, genetic data show that incorporation of new H3 increases in the absence of Spt16 (Voth et al. 2014), which indicates that FACT is involved in maintenance of preexisting tetrasome. FACT temperature-sensitive mutants impede nucleosome deposition, as determined by an in vivo ChIP assay (Nguyen et al. 2013).

These genetic studies support the idea that FACT is a nucleosome assembly factor. Based on these contradicting data, it was proposed that FACT facilitates both nucleosome disassembly and assembly by holding the components of nucleosome in a ternary complex during transcription elongation, and reassembling nucleosomes as RNA polymerase II passes through (Jamai et al. 2009). Recently, Tsunaka and his colleagues found that hFACT does not bind to well assembled nucleosome (Tsunaka et al. 2016). In contrast, hFACT efficiently forms a complex with nucleosomes if these were reconstituted with two DNA fragments (33/112-bp), which results in H2A-H2B displacement. These two DNA fragments create two DNA blunt ends closed to N-terminal tail of H2B. Accordingly, they proposed that FACT facilitates nucleosome disassembly through interaction with H2A-H2B. However, it is still unknown if FACT is indeed able to facilitate nucleosome assembly, and the exact role of FACT in nucleosome dynamics requires further investigation.

Histone posttranslational modifications (PTMs) have potential roles in histone chaperone activity. For example, nucleosomes with H3K36me3 facilitate FACT recruitment to the actively transcribed chromatin template, which results in maintenance of nucleosome integrity (Carvalho et al. 2013). Similarly, histone ubiquitination (ub) plays an important role in gene transcription (Wu et al. 2014). Carey and colleagues have shown that H2BK120 ubiquitination facilitates FACT function in transcription, in the presence of other transcription factors such as p300, Mediator and SWI/SNF etc. (Pavri et al. 2006). Transcription levels are significantly increased in an in vitro transcription system in the presence of FACT. This suggests that histone ubiquitination has potential roles in the regulation of FACT assembly/disassembly activity. It was proposed that H2BK120ub

stimulates FACT function to remove H2A-H2B dimers in transcription elongation, which promotes gene transcription (Pavri et al. 2006).

FACT per se is also a target for posttranslational modifications, which play an important role in the regulation of its function. CK2 phosphorylates hFACT, and thus preventing FACT DNA-binding activity (Li et al. 2005). Dephosphorylation of FACT restores its DNA binding activity (Tsunaka et al. 2009). Dephosphorylated FACT interacts nonspecifically with the nucleosome. Therefore, in this study we used phosphorylated FACT as done in previous studies (Tsunaka et al. 2009).

Here, we reveal that hFACT binds to H3-H4 with ~13 nM affinity, which is similar to the binding affinity of FACT-h(H2A-H2B) (~17 nM). We also provide direct evidence that FACT binds a histone hexamer, which is comprised of one $(H3\text{-}H4)_2$ tetramer and one H2A-H2B dimer. FACT-H2A-H2B interaction facilitates FACT binding to H3-H4. Additionally, the H2B-H4 interaction at H2A-H2B docking site closed to its base is crucial for FACT interaction with a histone hexamer. This suggests that FACT promotes (H2A-H2B)-(H3-H4) interaction. By binding all histones, FACT facilitates tetrasome assembly, as well as H2A-H2B deposition onto tetrasome and hexasome, which results in nucleosome assembly. Thus, FACT is a nucleosome assembly factor, consistent with genetic data. In addition, mono-ubiquitination at either H2AK119 or H2BK120 does not affect FACT-(H2A-H2B) interaction and FACT assembly activity.

Additionally, we find that FACT tethers components of the nucleosome via interaction with H2A-H2B and tetrasomal H3-H4. Compared to nucleosome, DNA does not stably wrap around histones. FACT-H2A-H2B interaction and dimer-tetramer interaction are

indispensable for FACT binding nucleosome components. According to our biochemical data, we propose that interaction with H2A-H2B facilitates FACT binding H3-H4 in the context of tetrasome. Through interaction with tetrasomal H3-H4, FACT further promotes the interaction between H2A-H2B and H3-H4. In this way, FACT is able to tether the partial components of nucleosome, containing a histone hexamer and DNA. The current work provides mechanistic insight into how FACT regulates nucleosome structure.

2.3 Material and Methods

2.3.1 Reagents

Recombinant histones used in this study were either from *Xenopus laevis* or *Homo sapiens.* They were expressed and purified as described previously (Luger et al. 1999). H4T71C mutant was used for H3-H4 Alexa 488 labeling, while H2BT112C was used for H2A-H2B Atto 647N labeling. 147-bp or 207-bp '601' DNA was prepared as described previously. Nucleosomes with 147-bp or 207-bp DNA were reconstituted by salt dialysis (Dyer et al. 2004). H2AK119ub and H2BK120ub were prepared as previously described (Long et al. 2014).

2.3.2 FACT expression and purification

The purification process of human FACT full length or FACT ΔCTD complexes was adapted from previous work with minor changes (Winkler et al. 2011). FACT complexes were purified over a 5 ml prepacked HisTrap HP column (GE Healthcare), followed by 5 ml prepacked HiTrap Q HP column (GE Healthcare). The final step was a Superdex 200 10/300 size exclusion column (GE Healthcare). FACT was stocked in 150 mM NaCl, 20

mM Tris pH 8.0, 10% glycerol, 0.01% CHAPS, 0.01% octyl glucoside and 1 mM TCEP. CHAPS and octyl glucoside were used to avoid nonspecific protein-protein interaction.

2.3.3 Fluorescence-based Microplate assay

To determine the binding affinity for FACT and histones, fluorescence-based plate assays and fluorescence resonance energy transfer (FRET)-based competition assay were performed in 150 mM NaCl, 20 mM Tris pH 8.0, 10% glycerol, 0.01% CHAPS, 0.01% octyl glucoside and 1 mM TCEP. 384-well Sensoplate Plus microplates (Greiner Bio-One) for fluorescence-based plate assay were prepared as published before (Winkler et al. 2011). Labeled *Xenopus laevis* or *Homo sapiens* histone was kept at 2 nM. hFACT complex was titrated from 1 nM to 500 nM. The reaction mixture was incubated in a 384-well microplate for 10 mins at room temperature, and then scanned by Typhoon 8600 variable mode fluorimager. Fluorescence signal was quantified by ImageQuant^{TM}. The data was analyzed and fit by Graphpad Prism. To test if histone ubiquitination affects FACT-(H2A-H2B) interaction, the FRET-based competition assay was conducted in the same buffer as fluorescence-based plate assay. 100 nM *Xenopus laevis* H2A-H2B dimer and 20 nM FACT concentration were mixed, and incubated for 10 mins at room temperature. *Homo sapiens* WT H2A-H2B, H2AK119ub-H2B or H2A-H2BK120ub was then titrated ranging from 1 nM to 3 µM. The reaction mixture was incubated in a 384-well microplate for 10 mins at room temperature, and then scanned by Typhoon. Fluorescence signal was quantified by ImageQuant^{TM}. The data was analyzed and fit with Graphpad Prism.

2.3.4 Sedimentation velocity analytical ultracentrifugation

To determine the stoichiometry of FACT-(H2A-H2B) complex, AUC was performed in 150 mM NaCl, 20 mM Tris pH 8.0 and 1 mM TCEP. 1.8 µM FACT was mixed with different amounts of H2A-H2B. The reaction mixture was kept at room temperature for 10 mins. The reaction mixture was spun at 30-35,000 rpm and 20 °C in a Beckman XL-A ultracentrifuge. To determine if FACT binds nucleosome, 150 nM nucleosome was mixed with 600 nM FACT, and incubated at room temperature for 10 mins. The reaction mixture was spun in either An-50 Ti or An-60 Ti rotor at 32,000 rpm 20 °C in a Beckman XL-A ultracentrifuge. All of AUC data was analyzed by UltraScan 3 version 2.0 and plotted by using Graphpad Prism.

To determine if FACT binds a histone hexamer, Sedimentation Velocity Analytical Ultracentrifugation (SV-AUC) experiments were performed using a Beckman Coulter Optima XL-A analytical ultracentrifuge equipped with an Aviv fluorescence detection system (FDS) using an An60Ti rotor (Beckman Coulter) with standard epon 2-channel centerpiece cells. Samples were diluted to desired concentrations ranging from ~0.2 – ~0.5 OD (absorbance), or Alexa 488 labeled histones were set to either 100 or 200 nM concentration (fluorescence). Sedimentation was then monitored using either the absorbance optics (intensity mode at 280 nm) or fluorescence optics (emission 488, excitation >505) at 20 °C using speeds of 40 or 45k rpm. Partial specific volumes of sample were determined using UltraScan 3 version 2.0. Time invariant and radial invariant noise were subtracted from the sedimentation velocity data by 2-dimensional-spectrum analysis (2DSA) followed by genetic algorithm refinement and Monte Carlo analysis (Brookes et al. 2010, Brookes and Demeler 2007, Demeler and Brookes 2008).

Sedimentation coefficient distributions G(s) were obtained with enhanced van Holde-Weischet analysis (Demeler and van Holde 2004).

Calculations were performed on the UltraScan LIMS cluster at the Bioinformatics Core Facility at the University of Texas Health Science Center at San Antonio and the Lonestar cluster at the Texas Advanced Computing Center supported by NSF Teragrid Grant #MCB070038.

2.3.5 In vitro gel-based assembly/disassembly assay

To test if FACT disassembles nucleosome, FACT (5 nM to 5 μM) was titrated into nucleosome previously assembled onto 147-bp DNA (10 nM). The reaction mixture was incubated at room temperature for 1 hr. The reaction was performed in the same buffer as mentioned above. Nucleosome was analyzed a 5% PAGE at 150 V, 4 ˚C for 60 mins.

To determine if FACT facilitates tetrasome assembly, FACT (15 nM to 240 nM) was titrated into *Xenopus laevis* or *Homo sapiens* H3-H4 (30 nM), and incubated at room temperature for 10 mins. DNA (30 nM) was added into the reaction mixture. The reaction was performed in the same buffer as mentioned above. Tetrasome formation was analyzed as mentioned above.

To determine if FACT facilitates H2A-H2B deposition onto tetrasome, Atto 647N labeled H2A-H2B dimer (30 nM) was mixed with varying amounts of FACT, and incubated at room temperature for 10 mins. 60 nM tetrasome was added into reaction mixture, and incubated at room temperature for another 30 mins. The reaction was performed in the same buffer as mentioned above. Products were analyzed as mentioned above.

To determine if FACT facilitates H2A-H2B deposition onto hexasome, under-assembled nucleosome was reconstituted with optimal ratio of H3-H4 to DNA and insufficient H2A-H2B. Under-assembled nucleosome was analyzed on a 5% PAGE. FACT (10 nM to 1280 nM) was titrated into under-assembled nucleosome (20 nM). The reaction mixture was incubated at room temperature for 1 hr. The reaction was performed in the same buffer as mentioned above. The reaction products were analyzed on a 5% PAGE at 150 V 4 ˚C for 70 mins.

To investigate if histone ubiquitination affects FACT assembly activity, under-assembled nucleosomes with ubiquitinated histones were reconstituted as WT under-assembled nucleosome. FACT (30 nM to 1920 nM) was titrated into under-assembled WT or modified nucleosome (20 nM). The reaction mixture was incubated at room temperature for 1 hr. The reaction was performed in the same buffer as mentioned above. The reaction products were analyzed as mentioned above.

To investigate if FACT facilitates nucleosome assembly, Alexa 488 labeled $(H3\text{-}H4)_2$ tetramer (250 nM) and Atto 647N labeled H2A-H2B dimer (500 nM) were mixed with or without FACT (1 µM), incubated at room temperature for 10 mins. Then, 207-bp '601' DNA (250 nM) was added into the reaction mixture, and incubated at room temperature for another 30 mins. The reaction was performed in 150 mM NaCl, 20 mM Tris pH 8.0, 10% glycerol, 0.01% CHAPS, 0.01% octyl glucoside and 1 mM TCEP. The reaction products were analyzed as mentioned above.

To determine if FACT assembles supershifted complexes with tetrasome with either 147 or 79-bp DNA, 850 nM FACT was mixing with an increasing amount of H2A-H2B (160 nM

and 240 nM), and then 40 nM tetrasome with either 147 or 79-bp DNA was added into reaction mixture. Complexes were analyzed as mentioned above.

2.3.6 Micrococcal nuclease digestion assay

To determine if histones are tightly wrapped by DNA in the supershifted complex, MNase digestion assay was applied. 3.2 µM FACT was mixed with 200 nM $(H3\text{-}H4)_2$ tetramer and 400 nM H2A-H2B dimer, incubated at room temperature for 10 mins. 100 nM 147-bp DNA was then added into the reaction mixture, and incubated at room temperature for another 30 mins. The reaction was performed in 150 mM NaCl, 20 mM Tris pH 8.0, 10% glycerol, 0.01% CHAPS, 0.01% octyl glucoside and 1 mM TCEP. Different amount of MNase (1 U/µl, 2 U/µl, 4 U/µl) was mixed with the supershifted complexes. The reaction mixtures were incubated at 37 °C for 10 mins. The reaction was quenched by adding 5 µl 0.5 M EDTA. 621-bp DNA was added into reaction mixture as a reference DNA. DNA fragments were purified through MiniElute PCR Purification Kit (QIAGEN). The length of DNA fragments was quantified by 2100 Bioanalyzer (Agilent), analyzed by 2100 Expert software. To determine the stability of nucleosome assembled by FACT, MNase digestion assay for nucleosome assembled by FACT was performed and analyzed as mentioned above.

2.3.7 In vitro transcription assay

In vitro transcription assay was adapted from previous work (Kuryan et al. 2012). 141-bp '601' DNA sequences, flanked by 50-bp polylinker DNA sequences and 20-bp DNA sequences were joined to a single-stranded C tail as a binding site for RNA pol II. Mononucleosomes were reconstituted onto this DNA fragment with *Xenopus laevis*

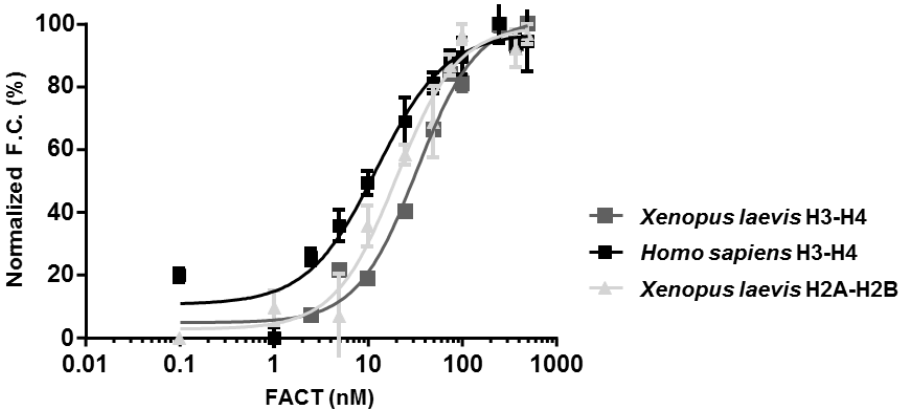
histones by salt dialysis; special care was taken to reach the correct assembly rate. All transcription reactions were performed in 25 mM HEPES pH 7.5, 10 mM $MgCl_2$, 2.5 mM KCl, 10% Glycerol, 1 mM DTT, and 250 ng/µl BSA. Nucleosome concentration was kept at 0.5 nM. FACT was titrated from 0.4 nM to 100 nM. Upon addition of NTPs and RNase H, chromatin template was transcribed by RNA pol II. The RNA transcripts are analyzed on a 6.5% acrylamide sequencing gel. RSC and RNA pol II were prepared as previously described (Izban and Luse 1991).

2.4 Results

2.4.1 FACT binds to a histone hexamer composed of one H2A-H2B and one $(H3\text{-}H4)_2$ tetramer.

The binding affinity of hFACT for hH3-H4 was determined using a fluorescence-based plate assay (Hieb et al. 2012). We found that hFACT binds hH3-H4 with an affinity of 13 nM (Figure 2.1A). This latter result is in disagreement with our previous work (Winkler et al. 2011), possibly due to differences in the preparation of FACT. In the current work, FACT was purified over an additional HisTrap Q HP column, in order to remove DNA contamination. We speculate that DNA contamination in previous FACT prep may result in nonspecific interactions between FACT and H3-H4. FRET-based competition assay was used to measure the binding affinity of hFACT with hH2A-H2B. hFACT binds hH2A-H2B with Kd=~17 nM affinity (Figure 2.1B). All these data support the idea that FACT is not only a H2A-H2B chaperone, but also a H3-H4 chaperone. Mono-ubiquitination at either H2AK119 or H2BK120 does not affect FACT-(H2A-H2B) interaction (Figure 2.1B). The stoichiometry of the hFACT-H2A-H2B complex was determined by Analytical

A


Normalized F.C. (%)
100
80
60
40
20
0
0.01
0.1
1
10
100
1000
FACT (nM)
Xenopus laevis H3-H4
Homo sapiens H3-H4
Xenopus laevis H2A-H2B

B

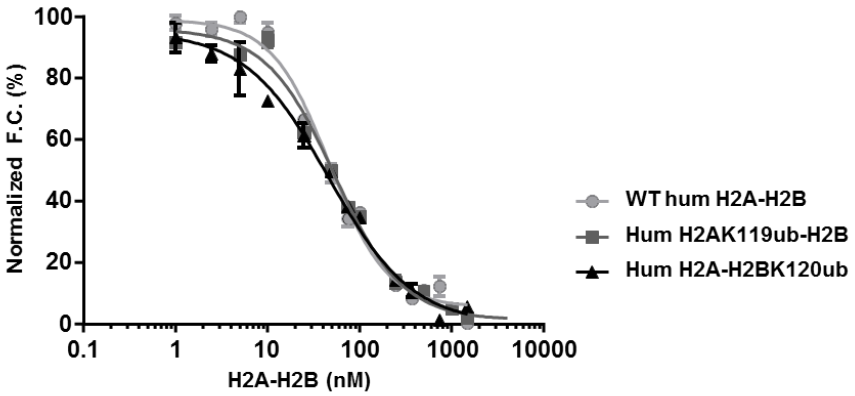


Figure 2.1. FACT binds to H3-H4 and H2A-H2B with similar affinity. (A) The binding affinity of FACT-H3-H4 was determined by fluorescence-based plate assay. *Xenopus laevis* and *Homo sapiens* H3-H4T71C were labeled with Alexa 488. *Xenopus laevis* H2A-H2BT112C was labeled with Atto 647N. H3-H4 and H2A-H2B concentration were kept at 2 nM. FACT concentration ranged from 1 nM to 500 nM. The reaction was performed under physiological salt condition (150 mM NaCl). The fluorescence signal is increased as a result of direct interaction between FACT and H3-H4 or H2A-H2B. The experiment was performed in three biological replicates. Each error bar represents standard deviation (SD) of three biological replicates (B) The contribution of mono-ubiquitination to FACT-H2A-H2B interaction was determined by FRET-based competition assay. *Xenopus laevis* H2A-H2BT112C was labeled with Alexa 488, and FACT was labeled with Atto 647N. Either WT or ubiquitinated human H2A-H2B were titrated from 1 nM to 3 μM. The FRET signal decreases as a result of competitive binding of histones to FACT. Each error bar represents SD of two biological replicates.

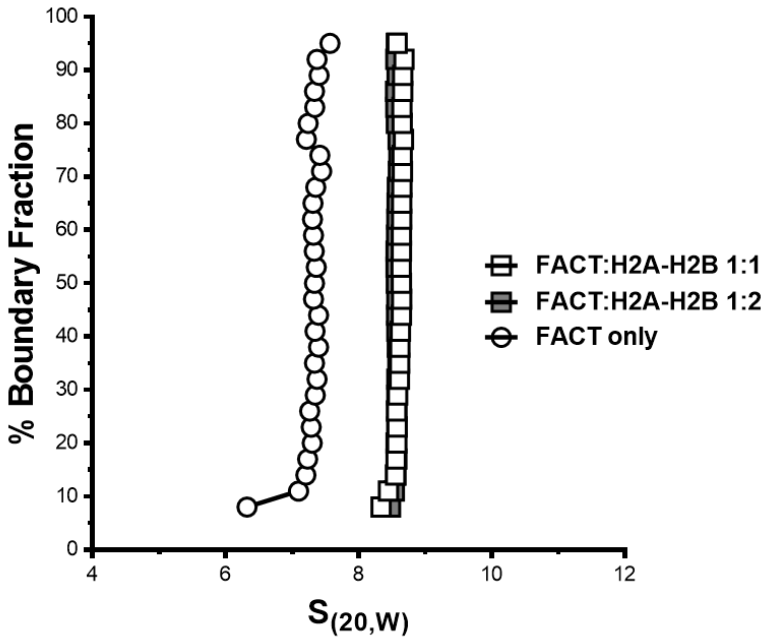


Figure 2.2. FACT binds one H2A-H2B dimer, and forms aggregates with $(H3\text{-}H4)_2$ tetramer at higher concentration. FACT concentration was kept at 1.8 μM, and mixed with histones at room temperature. H2A-H2B and FACT were pre-mixed at the indicated ratios. FACT forms aggregation with H3-H4 immediately as FACT is mixed with H3-H4, resulting in rapid loss of OD.

Ultracentrifugation (AUC) (Figure 2.2). The sedimentation coefficient ($S_{(20,W)}$) of hFACT alone is ~7.5. Addition of equimolar amounts of H2A-H2B increases the $S_{(20,W)}$ value of FACT to ~8.5, indicative of FACT-H2A-H2B interaction. Adding twice the amount of H2A-H2B does not further increase $S_{(20,W)}$ value of FACT-(H2A-H2B). The $S_{(20,W)}$ value of H2A-H2B is ~2.5 (Figure 2.3). This indicates that hFACT binds only one H2A-H2B dimer, which is in agreement with previous results (Winkler et al. 2011). The MW of FACT alone calculated $S_{(20,W)}$ by AUC is ~190 KDa, which is similar to its theoretical MW (201 KDa). The MW of FACT-H2A-H2B complex calculated in the same way is 214 KDa (theoretical MW is 228.9 KDa). We attempted to use this same approach to determine the stoichiometry of FACT-(H3-H4). An equimolar amount of $(H3\text{-}H4)_2$ tetramer (at a concentration of 1.8 µM) was mixed with FACT. Surprisingly, FACT-$(H3\text{-}H4)_2$ forms aggregates upon mixing. 1.8 µM $(H3\text{-}H4)_2$ was used in AUC experiment, which is 900-fold higher than the concentration used in the fluorescence-based plate assay. Perhaps the high concentration of H3-H4 causes nonspecific interactions with FACT, resulting in aggregation. Indeed, it has been shown that the acidic domain in Spt16 CTD interacts with H3-H4 nonspecifically (Tsunaka et al. 2016). In addition, H3-H4 exists as tetrameric state as H3-H4 concentration is high (>1 µM). As H3-H4 concentration is low (<50 nM), H3-H4 mainly exists as a dimeric state (Winkler et al. 2012). Thus, in the previous fluorescence-based plate assay H3-H4 exists as a dimer, while H3-H4 exists as a tetramer in AUC experiment. Likely, FACT prefers to bind to H3-H4 dimer, not H3-H4 tetramer.

It has been established that H2A-H2B binds to a conserved peptide motif of Spt16-CTD, while $(H3\text{-}H4)_2$ tetramer interacts with hSpt16-M domain (Kemble et al. 2015, Tsunaka et

al. 2016). We therefore asked if FACT can bind to H2A-H2B and H3-H4 simultaneously. To explore this idea, analytical ultracentrifugation was performed with an Aviv Fluorescence Detection System (AUC-FDS) to specifically monitor fluorescently labeled H2A-H2B. Alexa 488 labeled H2A-H2B concentration was kept at relatively low concentration (100 nM). FACT concentration was kept at 200 nM. The $S_{(20,W)}$ value of H2A-H2B alone is about ~2.5, whereas the $S_{(20,W)}$ value of H2A-H2B increases to ~8.3 in the presence of FACT, due to physical FACT-(H2A-H2B) interaction (Figure 2.3). This result is consistent with the AUC absorption data shown in Fig. 2.2. Addition of the fluorescence dye at H2B may affect S-value of H2A-H2B. To test if FACT binds to H2A-H2B and H3-H4 simultaneously, 400 nM FACT was mixed with 400 nM H3-H4 and 100 nM Alexa 488 labeled H2A-H2B dimer. Sedimentation coefficient distribution contains two species. One can be attributed to FACT-(H2A-H2B) complex ($S_{(20,W)}$=~8.3). The $S_{(20,W)}$ value of the other species is ~13.3, which we attribute to a FACT-$(H3\text{-}H4)_2$-(H2A-H2B) complex (Figure 2.3). We also attempted to determine the $S_{(20,W)}$ value of FACT-(H3-H4) by AUC-FDS. Alexa 488 labeled H3-H4 (at 200 nM) was combined with 200 nM FACT. Again, FACT and H3-H4 form fast-sedimenting aggregation, which causes loss of ~ 50% of the fluorescence intensity signal (Figure 2.4). In presence of H2A-H2B, we were able to regain this the lost signal. Furthermore, an $S_{(20,W)}$ value of FACT-$(H3\text{-}H4)_2$-(H2A-H2B) ($S_{(20,W)}$=~13.8) was obtained by using fluorescently labeled H3-H4 (Figure 2.3). Likely, H2A-H2B facilitates FACT interaction with H3-H4 by preventing nonspecific interaction between FACT and H3-H4. We attempted to calculate the MW of FACT and hexamer complex by AUC. However, the derived molecular weight is 499 KDa, which is much larger than its theoretical MW 279 KDa expected for FACT-hexamer complex. This may

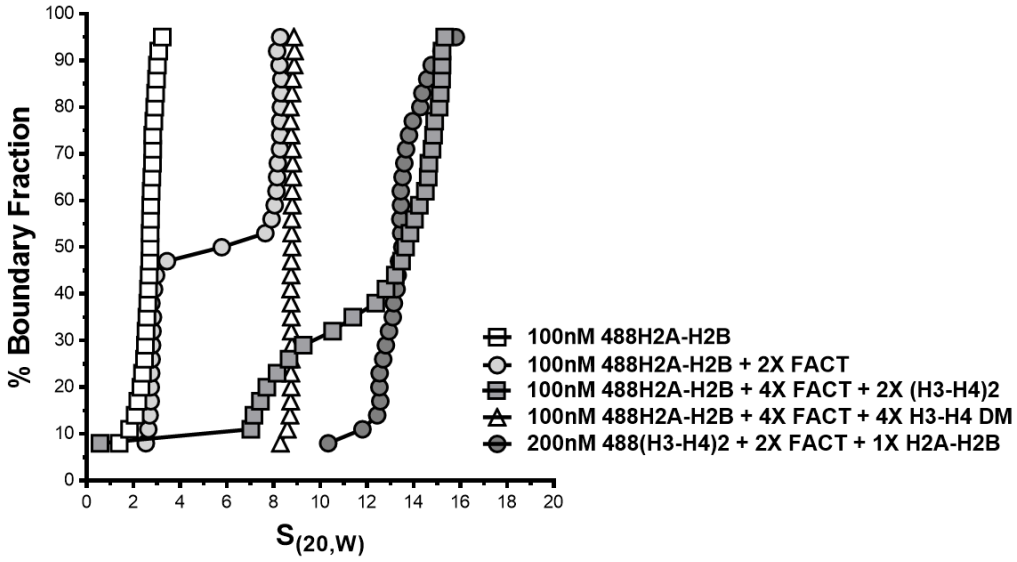


Figure 2.3. FACT binds to a histone hexamer composed of one $(H3\text{-}H4)_2$ tetramer and one H2A-H2B dimer. AUC with Fluorescence Detection System (FDS; detection at 488 nm) was performed for H2A-H2B only, FACT-(H2A-H2B) and FACT-$(H3\text{-}H4)_2$-(H2A-H2B). 100 nM Alexa 488 labeled H2A-H2B was mixed with 200 nM FACT. In presence of FACT, $S_{(20,W)}$ value of labeled H2A-H2B increased to ~8.3, due to FACT-H2A-H2B complex formation. This result is consistent with AUC absorption data (Fig. 2.2). In order to determine if FACT binds H2A-H2B and H3-H4 simultaneously, 100 nM Alexa 488 labeled H2A-H2B was mixed with 200 nM $(H3\text{-}H4)_2$ tetramer and 400 nM FACT. Addition of H3-H4 further increases the $S_{(20,W)}$ value of FACT-(H2A-H2B) from ~8.3 to ~13.3. This indicates that FACT binds H2A-H2B and H3-H4 simultaneously. Mutant H3-H4 tetramer no longer capable of forming a four-helix bundle (H3L126A, I130A and C110E), and thus are deficient in tetramer formation, was also analyzed. Besides, the S-value of FACT-$(H3\text{-}H4)_2$-(H2A-H2B) was determined by using Alexa 488 labeled H3-H4.

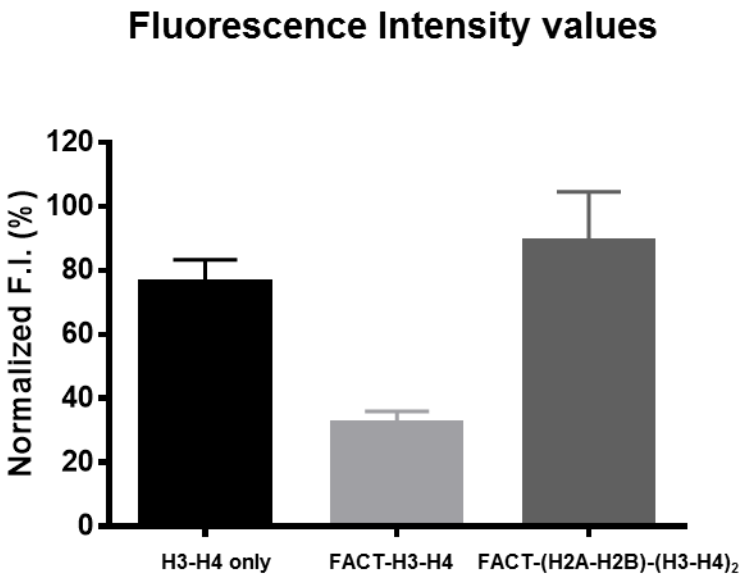


Figure 2.4. H2A-H2B prevents FACT from forming aggregates with H3-H4. We attempted to determine the $S_{(20,W)}$ value of FACT-H3-H4 by using AUC-FDS. Even at H3-H4 concentration around 200 nM, FACT still forms aggregation with H3-H4, which causes ~50% signal loss. In presence of H2A-H2B, the lost signal is regained, which suggests that H2A-H2B facilitates the formation of an ordered complex between FACT and H3-H4. The error bars represent SD of two biological replicates.

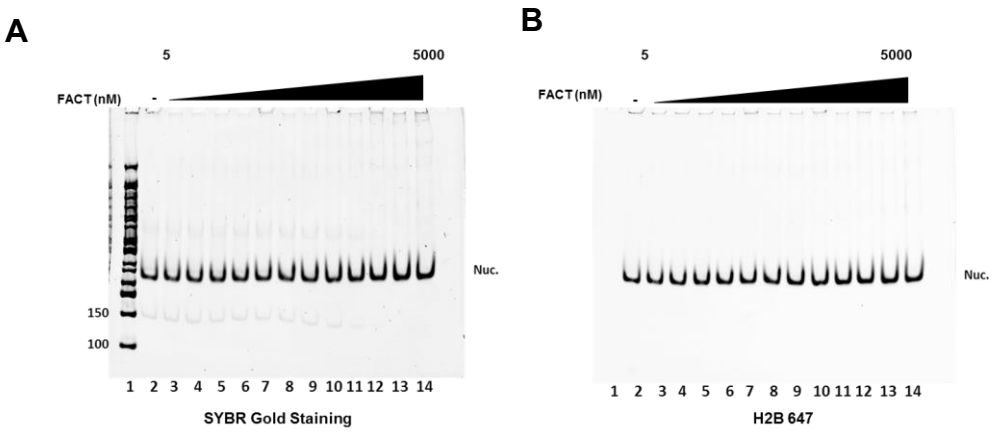
be due to the fact that partial specific volume (i.e. the volume that the solute occupies in solution) is determined of f/f0 (gross shape and molecular weight). Partial specific volume was determined by using UltraScan 3 version 2.0, which cannot be precisely estimated for heterogeneous mixtures. Thus, MW of FACT-hexamer complex calculated by AUC is not similar to its theoretical MW.

In the previous experiment, H3-H4 concentration was kept below 500 nM, and thus H3-H4 exists as a mixture of H3-H4 dimer and $(H3\text{-}H4)_2$ tetramer. It is thus possible that FACT only binds one H3-H4 dimer and one H2A-H2B dimer simultaneously. In order to rule out this possibility, H3 with three point mutations (L126A, I130A, C110E) was used. According to the crystal structure of Spt16-MD-AID-$(H3\text{-}H4)_2$, these point mutations are not located at the interface of FACT-$(H3\text{-}H4)_2$ tetramer interaction. At 2 M NaCl, these mutations prevent the formation of the $(H3\text{-}H4)_2$ tetramer (data not shown); rather, H3-H4 forms a heterodimer. 400 nM FACT was mixed with 400 nM H3-H4 with the three point mutations and 100 nM Alexa 488 labeled H2A-H2B. Addition of H3-H4 dimer mutant does not increase $S_{(20,W)}$ value of the FACT-H2A-H2B (Figure 2.3). This indicates that FACT does not tether H3-H4 dimer with H2A-H2B dimer simultaneously. In sum, FACT binds a histone hexamer composed of one $(H3\text{-}H4)_2$ tetramer and one H2A-H2B dimer.

Together, we found that FACT binds H2A-H2B and H3-H4 with similar affinity, mono-ubiquitination at either H2A or H2B does not disrupt FACT-H2A-H2B interaction. In addition, H2A-H2B facilitates FACT interaction with $(H3\text{-}H4)_2$ tetramer, which results in the formation of a FACT-histone hexamer complex, comprised of one $(H3\text{-}H4)_2$ tetramer and one H2A-H2B dimer.

2.4.2 FACT per se does not either disassemble or bind to nucleosome in vitro.

FACT was originally identified as a nucleosome disassembly factor. It was suggested that FACT removes one or two H2A-H2B dimers from nucleosomes (Belotserkovskaya et al. 2003). This is actually a reasonable assumption in light of the high FACT-H2A-H2B binding affinity. To test this hypothesis, an in vitro gel-based nucleosome disassembly assay was performed. Excess amounts of FACT were mixed with nucleosomes reconstituted onto a 147-bp DNA fragment by salt dialysis. Reaction products were analyzed on a 5% PAGE, and visualized by SYBR gold staining and fluorescence (H2B) (Figure 2.5A-B). The intensity of the nucleosome band remains constant as FACT is titrated. Thus, FACT per se does not disassemble nucleosome. Additionally, no FACT-nucleosome complexes were observed on the gel. The inability of FACT to bind fully assembled nucleosomes was confirmed by AUC. The $S_{(20,W)}$ value of a mononucleosome is about ~11. Addition of 4-fold excess of FACT does not alter the $S_{(20,W)}$ value of mononucleosome (Figure 2.5C), indicating that FACT does not bind to the nucleosome. According to sedimentation coefficient distribution, no sub-nucleosome complexes were observed, confirming that FACT does not disassemble nucleosomes. This result is consistent with previously published data (Tsunaka et al. 2016). However, it is not consistent with our previous work (Winkler et al. 2011). It may be due to the fact that the binding affinity of FACT-nucleosome was determined by fluorescence-based plate assay. In the previous work, nucleosome with fluorescently labeled H2A-H2B was applied. The binding event could be due to FACT interaction with free labeled H2A-H2B in the reaction mixture.


A
5
5000
FACT (nM)
-
Nuc.
150
100
1 2 3 4 5 6 7 8 9 10 11 12 13 14
SYBR Gold Staining
B
5
5000
FACT (nM)
-
Nuc.
1 2 3 4 5 6 7 8 9 10 11 12 13 14
H2B 647

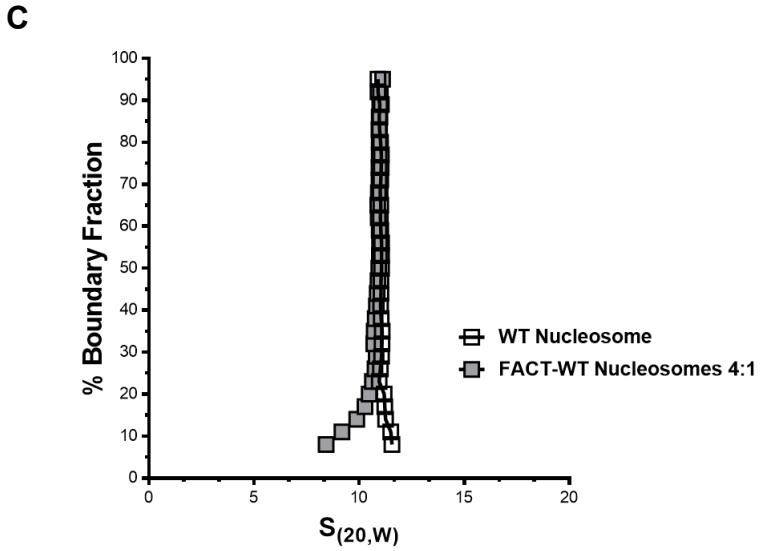


Figure 2.5. FACT does not disassemble or interact with properly assembled nucleosomes. (A; B) Nucleosomes were reconstituted with 147-bp '601' DNA and histone octamer (fluorescently labeled on H2B) using salt dialysis. Nucleosome concentration was kept at 10 nM. In absence of FACT, nucleosome is not disassembled at room temperature (lane 2). As FACT is titrated (from 10 nM to 5 μM), the amount of nucleosome remains constant (A) and (B), and no FACT-H2A-H2B complex was observed (B). Nucleosomes were analyzed on a 5% PAGE, visualized by SYBR Gold (A) and fluorescence (H2B) (B), and scanned by Typhoon. (C) In order to confirm that FACT does not bind nucleosome, AUC was applied. Nucleosome (130 nM) was mixed with 520 nM FACT at room temperature for 30 mins. AUC was performed at 20 °C, spun at 35K rpm. The reaction was performed in 150 mM NaCl, 20 mM Tris pH 8.0, 1 mM TCEP. Addition of FACT does not further increase $S_{(20,W)}$ value of nucleosome, which implies FACT does not bind to nucleosome. Based on the distribution of sedimentation coefficient, no sub-nucleosomal complexes were observed.

2.4.3 FACT is a robust tetrasome assembly factor, and facilitates H2A-H2B deposition onto tetrasomes and hexasomes.

Having shown that FACT binds all histones, we next investigated whether FACT could assemble nucleosomes. First, the role of FACT in tetrasome assembly was determined. An in vitro gel-based tetrasome assembly assay was performed. FACT was mixed with H3-H4, and then 147-bp '601' DNA was added into the reaction mixture. The reaction products were analyzed on a 5% PAGE, visualized by SYBR Gold staining (DNA complexes) (Figure 2.6). In absence of FACT, only a small amount of H3-H4 bound to DNA forms tetrasome (the slowest migrating band) (Figure 2.6 lane 3 and 9) (Flaus et al. 1996). In contrast, increasing amounts of tetrasome was assembled as FACT was titrated (Figure 2.6 lane 4-8 and lane 10-14). No significant difference between *Xenopus laevis* and *Homo sapiens* H3-H4 was observed.

Subsequently, we asked if FACT facilitates H2A-H2B deposition onto tetrasomes, by employing an in vitro gel-based nucleosome assembly assay as above. Tetrasome was reconstituted from 147-bp '601' DNA and H3-H4 by salt dilution. H2A-H2B was mixed with different amounts of FACT, and then tetrasome was added into the reaction mixture. Products were analyzed on a 5% PAGE, visualized by SYBR Gold staining (DNA complexes) (Figure 2.7). To some extent, H2A-H2B deposits onto tetrasome in absence of FACT (Figure 2.7A-B lane 3). In contrast, fluorescence signal from hexasomal and nucleosomal DNA and H2B is increased as FACT is titrated (Figure 2.7A-B, lanes 8-11). This implies that FACT facilitates H2A-H2B to bind tetrasomes, thereby promoting the assembly of both hexasomes and nucleosomes.

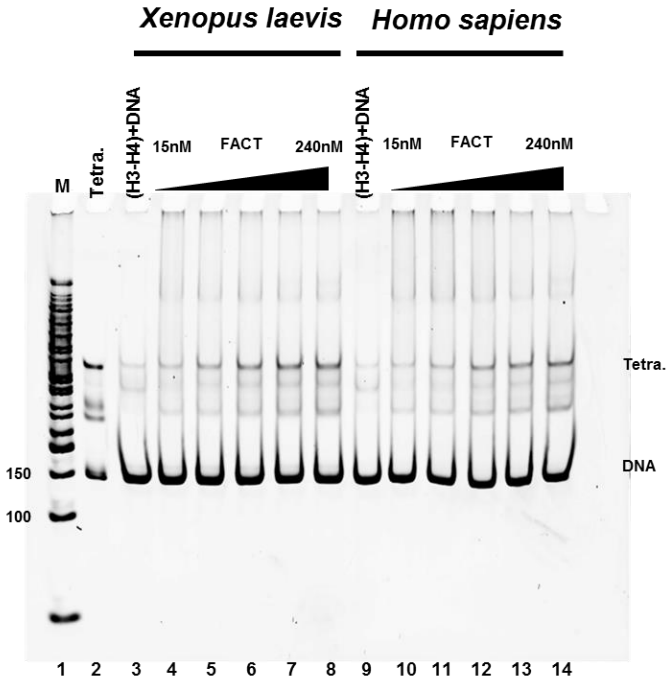


Figure 2.6. FACT facilitates tetrasome formation. Tetrasome assembly assay was performed with 147-bp '601' DNA and *Xenopus laevis* or *Homo sapiens* H3-H4. DNA and H3-H4 concentration were kept at 30 nM. FACT concentration was increased from 15 nM to 240 nM. FACT was mixed with H3-H4, and incubated for 30 mins at room temperature. Then, DNA was added into the reaction mixture, and incubated for another 30 mins at room temperature. Tetrasomes were analyzed on a 5% PAGE, and visualized by SYBR Gold (DNA complexes). The reaction was performed under physiological salt condition. Both *Xenopus laevis* and *Homo sapiens* H3-H4 bind to DNA and form tetrasome under physiological salt condition (lane 3 and 9). However, assembly efficiency is significantly increased in the presence of FACT (lane 4 to 8 and lane 10 to 14), Tetrasome in lane 2 is pre-reconstituted with 147-bp '601' DNA and *Xenopus laevis* tetramer by salt dialysis.

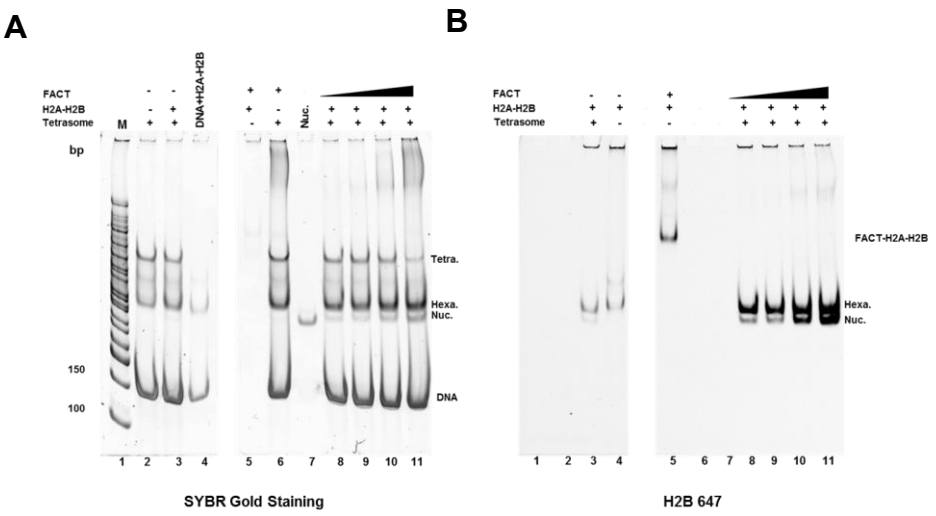
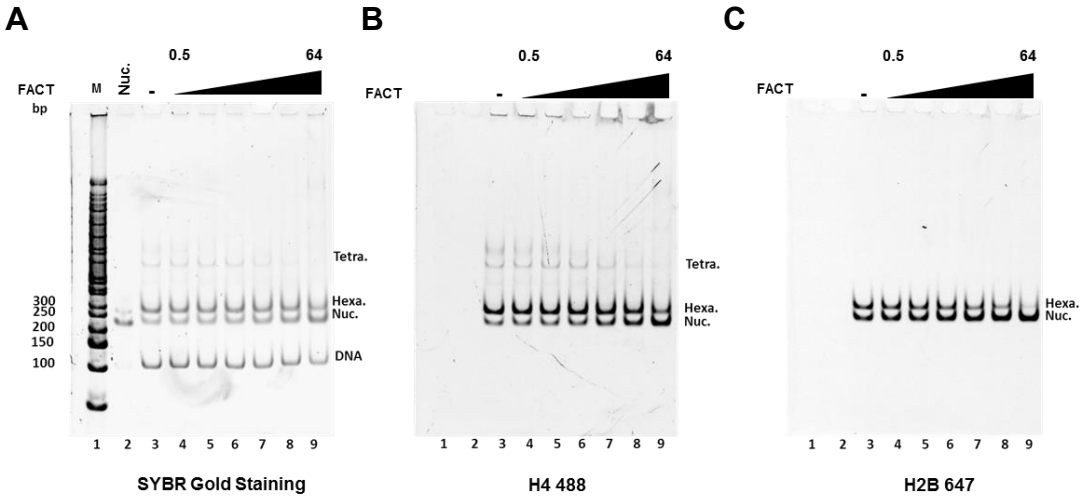


Figure 2.7. FACT facilitates the deposition of H2A-H2B onto the tetrasome, resulting in hexasome and nucleosome assembly. Salt-reconstituted tetrasome (lane 2) and Atto 647N labeled H2A-H2B were incubated with increasing amounts of FACT. Tetrasome and H2A-H2B concentration were kept at 60 nM and 30 nM, respectively. FACT (30 nM to 1920 nM) was incubated with H2A-H2B at RT for 30 mins, and then pre-assembled tetrasome was added. Nucleosomes were analyzed on a 5% PAGE, and visualized by SYBR Gold (DNA complexes; A), or fluorescence (H2B; B). H2A-H2B binds to tetrasome under physiological salt condition (A, lane 3), but the amount of nucleosome or hexasome band is increased with increasing amounts of FACT, whereas the signal of tetrasomal DNA is decreased (A lane 8 to 11). Lane 2, 4 to 7 (A) are loading controls for tetrasome, DNA-dimer complex, FACT-dimer complex, FACT-tetrasome complex and nucleosome.

We next wanted to investigate whether the hexasome is also a substrate for FACT-mediated nucleosome assembly. Under-assembled nucleosomes were reconstituted by mixing optimal amount of DNA and H3-H4, but insufficient H2A-H2B. In this way, hexasomes were the major components in the solution. The hexasome is an intermediate state between tetrasome and nucleosome. Thus, a reaction equilibrium is reached between nucleosomes, hexasomes and tetrasomes. In other words, the reaction mixture (Figure 2.8A-C, lane 2) may contain small amount of free H2A-H2B. In order to examine if FACT facilitates the deposition of H2A-H2B onto hexasomes, under-assembled nucleosome was mixed with increasing amounts of FACT. Nucleosomes, hexasomes and tetrasomes were analyzed on a 5% PAGE, visualized by SYBR Gold (DNA complexes), fluorescence (H4) or fluorescence (H2B) (Figure 2.8A-C). The amount of nucleosome, tetrasome and hexasome was determined by ImageQuant^{TM} (Figure 2.8D-F). As FACT is titrated, the fluorescence signals from nucleosomal H4 is increased, while the signal from tetrasomal H4 decreases (Figure 2.8D and E). In contrast, fluorescence signal from hexasomal H2B increases, and then decreases during the FACT titration series (Figure 2.8F). This suggests that FACT facilitates the deposition of H2A-H2B dimer to form nucleosomes from under-assembled nucleosomes. The role of mono-ubiquitination of either H2A or H2B in FACT-mediated nucleosome assembly activity was also determined. Although mono-ubiquitination at either H2AK119 or H2BK120 does not affect FACT-(H2A-H2B) interaction (Figure 2.1B), we wanted to test whether these modifications affect FACT nucleosome assembly activity. To address this question, under-assembled nucleosome with either WT or modified histones was mixed with increasing amounts of FACT. As FACT is titrated, the level of nucleosome with either H2AK119ub-H2B or


A
B
C
FACT
M
Nuc.
-
0.5
64
bp
300
250
200
150
100
Tetra.
Hexa.
Nuc.
DNA
1 2 3 4 5 6 7 8 9
SYBR Gold Staining
H4 488
H2B 647

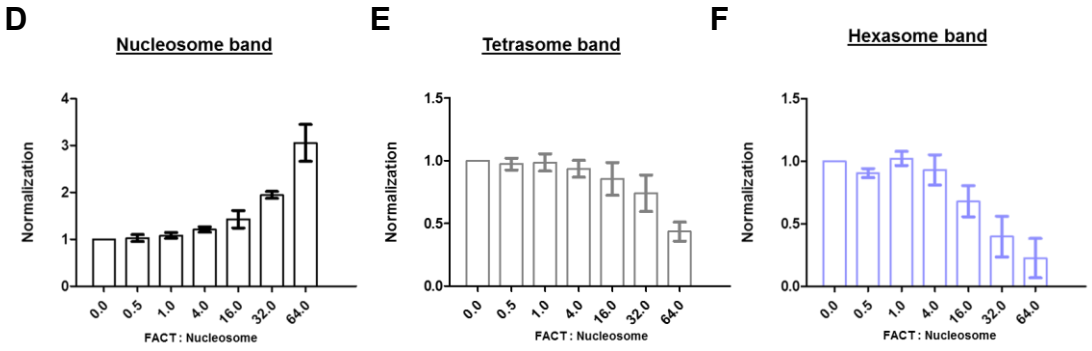


Figure 2.8. FACT facilitates the deposition of H2A-H2B onto hexasome. A) Under-assembled nucleosome (lane 3) was incubated with increasing amounts of FACT, and assembly products were analyzed on a 5% PAGE. The gel was scanned for fluorescence on a typhoon and visualized by SYBR Gold (DNA complexes; A), fluorescence (H4; B), or fluorescence (H2B; C). The fluorescence signals from nucleosomal H4 (D), tetrasomal H4 (E) and hexasomal H2B (F) were analyzed. The gel shown in B was quantitated by ImageQuant^{TM}. In the presence of FACT, the fluorescence signal for nucleosomal H4 is significantly increased (D), whereas the signal for tetrasomal H4 is decreased (E), All the bar graphs represent the average from three biological replicates (error bars represent SEM). Lane 2 (A) is a loading control for nucleosome.

H2A-H2BK120ub are increased as well (Figure 2.9A-B). Thus, mono-ubiquitination at either H2AK119 or H2BK120 does not have a positive or negative effect on FACT-mediated nucleosome assembly activity.

We next investigated whether FACT assembles nucleosomes *de novo,* that is, from histones and DNA. Increasing amounts of FACT was mixed with 50 nM $(H3\text{-}H4)_2$ tetramer and 100 nM H2A-H2B dimer, and then 25 nM DNA was added into the reaction mixture. H3-H4 and H2A-H2B were fluorescently labeled with Alexa 488 and Atto 647N, respectively. The reaction products were analyzed by 5% PAGE, and visualized by SYBR Gold staining, fluorescence (H4) and fluorescence (H2B) (Figure 2.10). Under these conditions, in absence of FACT, histones nonspecifically bind to DNA, and only a small amount of nucleosome is formed (the fastest migrating band) (Figure 2.10A-C lane 2). In contrast, an increasing amount of nucleosomes are assembled in presence of FACT (Figure 2.10A-C lane 6-10). Additionally, we note two complexes with much higher electrophoretic mobility in the presence of FACT. These two supershifted complexes containing H3-H4, H2A-H2B and DNA will be further discussed below (Figure 2.10A-C).

To confirm that nucleosomes assembled by FACT have similar stability as nucleosome reconstituted by salt dialysis, we compared their resistance towards micrococcal nuclease (MNase) digestion. The pause sites for MNase are caused by histone-DNA interaction. Nucleosome was assembled by 1 µM FACT with 250 nM $(H3\text{-}H4)_2$ tetramer, 500 nM H2A-H2B dimer and 250 nM 207-bp DNA. The supershifted complexes, as observed above, contain flag-tagged FACT and were removed by anti-FLAG affinity purification, and the flow through was collected for MNase digestion (Figure 2.11A). Nucleosomes reconstituted by salt dialysis or assembled with or without FACT were mixed with

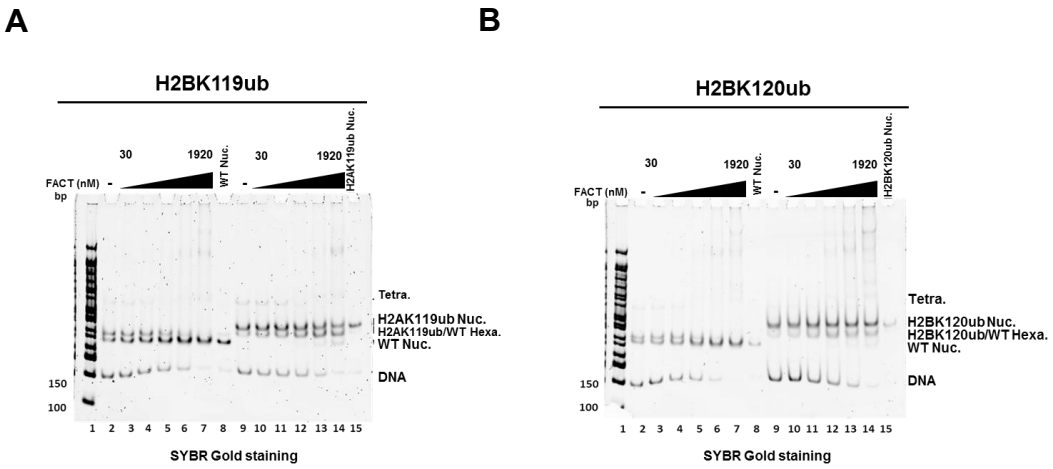


Figure 2.9. H2AK119ub and H2BK120ub do not affect FACT assembly activity. Under-assembled WT, H2AK119ub or H2BK120ub nucleosome were incubated with increasing amounts of FACT (from 30 nM to 1920 nM), and analyzed on a 5% PAGE. The gel was scanned by typhoon and visualized by SYBR Gold. FACT increases the amount of unmodified nucleosomes (A and B; lane 3-7). Similarly, the level of nucleosomes with either H2AK119ub or H2BK120ub are increased. Notably, hexasomes containing either H2AK119ub or H2BK120ub are increased as well (A and B lane 10-14), which suggests that monoubiquitination at either H2AK119 or H2BK120 does not affect FACT assembly activity.

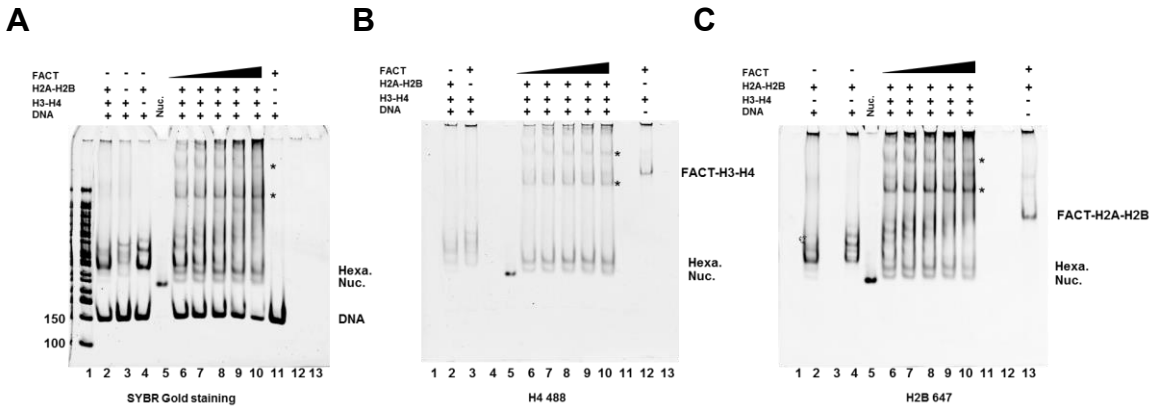
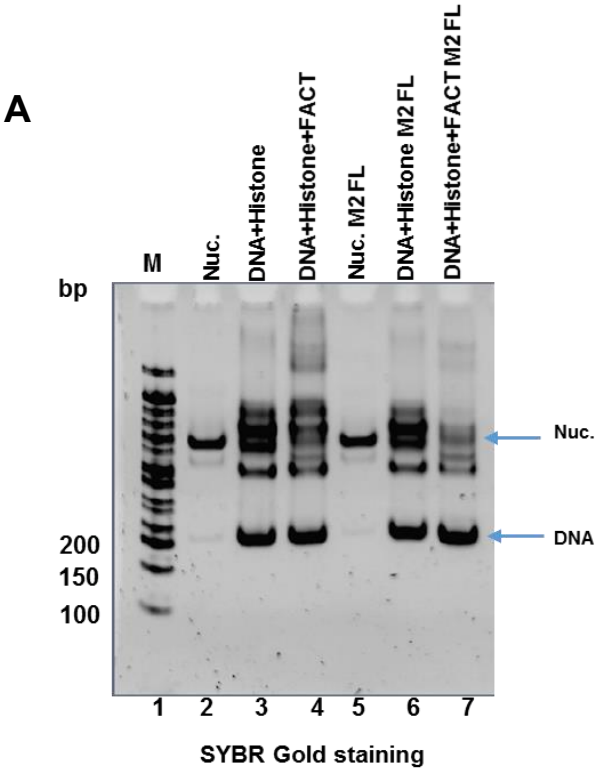


Figure 2.10. FACT facilitates nucleosome assembly *de novo*. 147-bp '601' DNA concentration was kept at 25 nM. $(H3\text{-}H4)_2$ and H2A-H2B concentration were kept at 50 nM and 100 nM, respectively. FACT was incubated with histones at RT for 10 mins, and then DNA was added. The reaction mixture was incubated at RT for another 30 mins. Nucleosomes and sub-nucleosome complexes were analyzed on a 5% PAGE, visualized by SYBR Gold (DNA complexes) (A), H4 fluorescence (B), or H2B fluorescence (C). In absence of FACT, low levels of interaction of histones with DNA were observed (lane 2). In presence of FACT, nucleosome and hexasome are assembled (A, B and C, lane 6 to 10). It suggests that FACT assembles nucleosome and hexasome. Additionally, we observed that two supershifted complexes (asterisked) are increased, as FACT is titrated. These complexes contain H2A-H2B, H3-H4 and DNA, which will be discussed later.

increasing amounts of MNase (100, 200 and 400 U). The reaction mixture was incubated at 37 °C for 10 mins. Protected DNA fragments were purified through MiniElute PCR Purification Kit (QIAGEN), and analyzed by Bioanalyzer (Agilent) (Figure 2.11B). Nucleosomes assembled by FACT with 400 U MNase exhibit an MNase pause site at 126-bp, very similar to what is observed for nucleosomes reconstituted by salt dialysis. In contrast, histones added to DNA in absence of FACT do not cause a significant pausing site at 126-bp, instead it causes a pause site at 98-bp (Table 2.1). Together, this data suggests that nucleosome assembled by FACT has similar stability to nucleosome reconstituted by salt dialysis. In sum, FACT has moderate nucleosome assembly activity via facilitating tetrasome assembly, and H2A-H2B deposition onto tetrasome and hexasome. Mono-ubiquitination at either H2AK119 or H2BK120 does not affect FACT assembly activity.

2.4.4 FACT forms two supershifted complexes with tetrasome and H2A-H2B.

The supershifted complexes were observed in the previous nucleosome assembly assay, which contain fluorescently labeled H4, fluorescence labeled H2B and DNA (Figure 2.10). Next, we tested if FACT is able to assemble the similar complexes with tetrasome and H2A-H2B. In order to address this question, tetrasome was reconstituted with 147 bp '601' DNA and H3-H4 by salt dilution. 850 nM FACT was mixed with 60 or 120 nM H2A-H2B, and then 40 nM tetrasome was added into the reaction mixture. The supershifted complexes were analyzed on a 5% PAGE, visualized by SYBR Gold staining (DNA complexes) (Figure 2.12). We noticed that FACT per se does not bind to tetrasome (Figure 2.12 lane 4). However, in presence of H2A-H2B, FACT binds tetrasome efficiently, and forms two supershifted complexes (Figure 2.12, lanes 5-6). It suggests that H2A-H2B


A
M
Nuc.
DNA+Histone
DNA+Histone+FACT
Nuc. M2 FL
DNA+Histone M2 FL
DNA+Histone+FACT M2 FL
bp
200
150
100
Nuc.
DNA
1 2 3 4 5 6 7
SYBR Gold staining

B

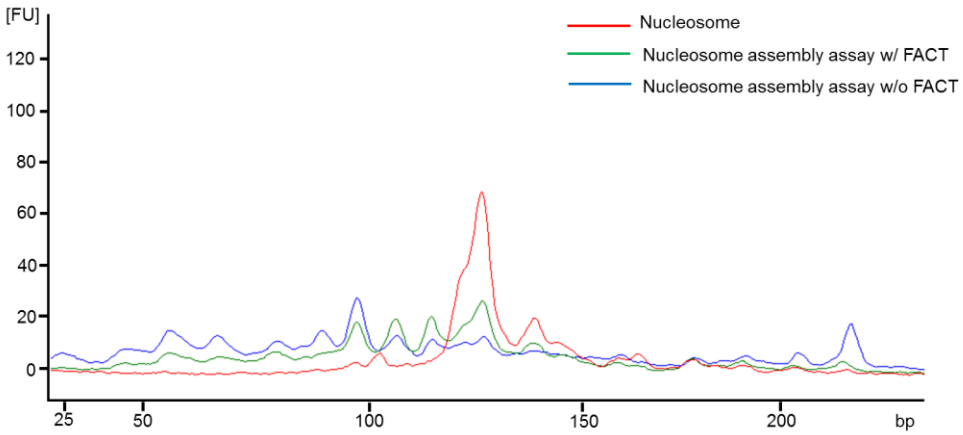


Figure 2.11. Nucleosomes assembled by FACT have similar resistance towards MNase as salt-reconstituted nucleosomes. 1 μM FACT was mixed with 250 nM H3-H4 tetramer and 500 nM H2A-H2B dimer, and then 250 nM 207-bp '601' DNA was added into reaction mixture. The supershifted FACT-containing complex was removed by M2 resin, and the flow through (containing various assembly products not bound by FACT) was collected for MNase digestion. Protected DNA fragments were quantified by Bioanalyzer. Nucleosome assembled by FACT (green trace) has similar MNase pausing site at 126-bp, compared to salt-reconstituted 207 nucleosome (red line). In contrast, histones combined with DNA at low salt, and in the absence of FACT (blue line) does not result in this typical digestion pattern.

Table 2.1. FACT facilitates nucleosome and sub-nucleosomal particle assembly. Recovery rate was calculated by total molarity of DNA fragments (400 U MNase) dividing by the molarity of total input DNA (no MNase). Since input DNA for histone-DNA with or without FACT mainly contains free DNA and sub-nucleosome particles (Fig 2.11), they are rapidly digested by 400U MNase. Thus, low recovery rate was obtained for histone-DNA with or without FACT.

Size (bp)	207 Nucleosome (MNase 400U)	207 DNA+Histone w/o FACT (MNase 400U)	207 DNA+Histone w/ FACT (MNase 400U)
	Molarity (nmol/l)	Molarity (nmol/l)	Molarity (nmol/l)
98	0	16.4	0
106	0	0	11.6
115	0	0	9.9
126	34.7	0	19.5
139	6.9	0	0
Recovery Rate	**74.7%**	**6.3%**	**16.9%**

facilitates FACT interaction with tetrasomes. We speculate that these two supershifted complexes are assembled due to FACT tethering nucleosome components. Besides, we did not observe that significant amount of supershifted complexes were assembled in the H2A-H2B deposition assay (Figure 2.7), where we show that FACT facilitates H2A-H2B deposition onto tetrasomes. Likely, it is due to the fact that more than 2-fold of H2A-H2B were used in this assay, which may result in increasing amount of FACT-H2A-H2B complex. Thus, FACT-H2A-H2B complex may be required for supershifted complexes assembly (Figure 2.12).

To further confirm that the supershifted complexes assembled by tetrasome and H2A-H2B also contain all histones, DNA and FACT, anti-FLAG tag affinity purification for FACT was performed. Two supershifted complexes were assembled by FACT, H2A-H2B and tetrasome, and purified through affinity chromatography. Supershifted complexes were analyzed on a 5% PAGE, and visualized by SYBR gold staining, fluorescence (H4), fluorescence (H2B) and overlay of H2B and H4 fluorescence (Figure 2.13). Nucleosomes and sub-nucleosomal complexes were enriched in the flow through, while only the supershifted complexes were eluted by FLAG peptide. We found that the complexes assembled by tetrasome, H2A-H2B and FACT also contain all histones, as well as DNA, and that these complexes are reasonably stable. In addition, the gel in Figure 2.13B and C was quantified by using ImageQuantTL. We found that the ratio between $(H3\text{-}H4)_2$ tetramer and (H2A-H2B) dimer in the lower complex is ~1.7, which is decreased to ~1.2 in the upper one. It indicates that the upper complex contains more H2A-H2B dimers than the lower one. Again, the second H2A-H2B may bind to the free tetrasomal DNA.

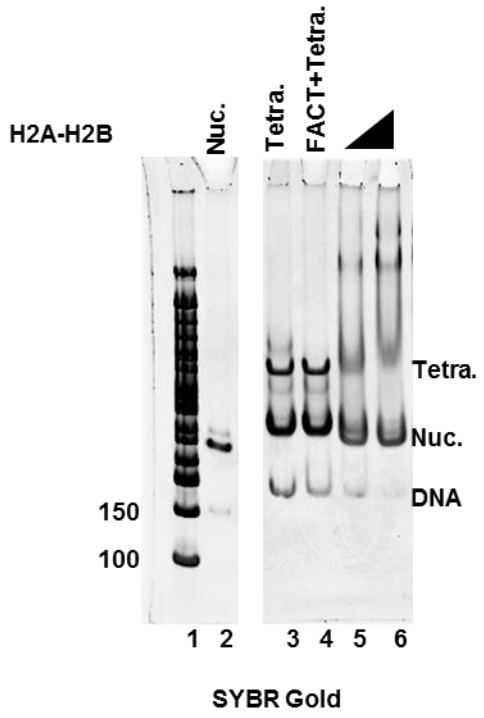


Figure 2.12. H2A-H2B facilitates FACT interaction with tetrasome, and forms two supershifted complexes. FACT was mixed with increasing amounts of H2A-H2B (60 nM and 120 nM), then tetrasome was added. Supershifted complexes were analyzed on a 5% PAGE, and visualized by SYBR Gold. FACT and tetrasome concentration were kept at 800 nM and 40 nM, respectively. It was observed that FACT binds to tetrasome in the presence of H2A-H2B, and forms two supershifted complexes (asterisks). The upper supershifted complex is formed as H2A-H2B concentration is high.

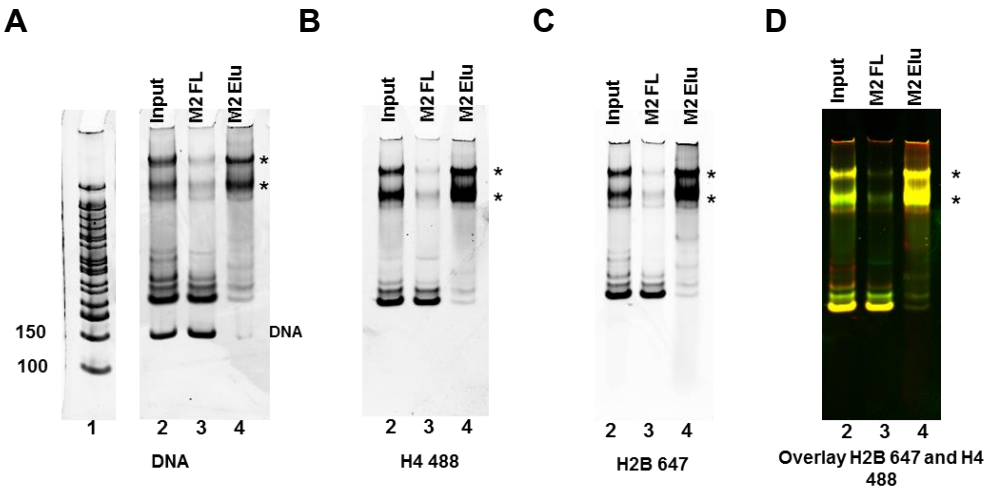


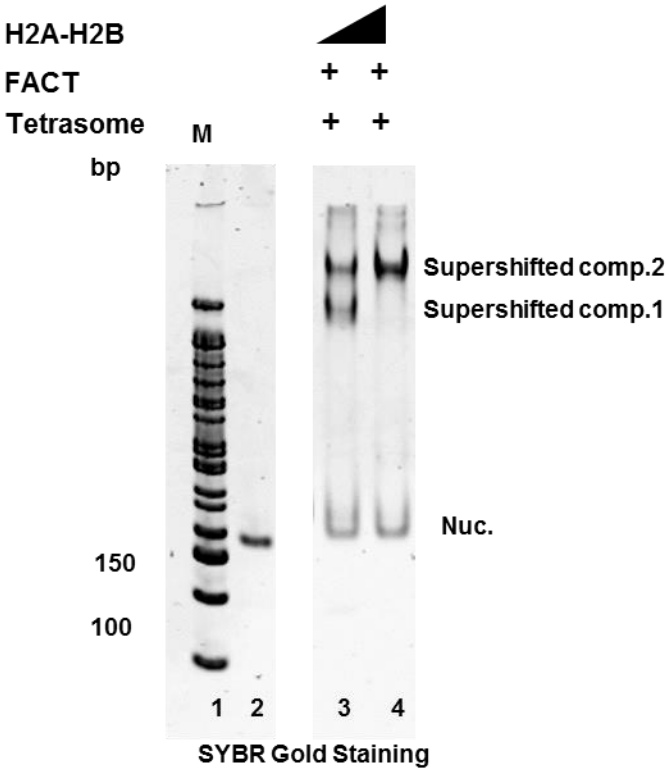
Figure 2.13. Supershifted complexes assembled by FACT, H2A-H2B and tetrasome are also comprised of FACT, all four histones, and DNA. To confirm that supershifted complexes assembled by tetrasomes, FACT and H2A-H2B also contain all histones and DNA, FACT was mixed with H2A-H2B first, and then tetrasome was added into reaction mixture. FLAG-tag affinity purification was performed. Alexa 488 labeled tetrasome concentration is ~150 nM. FACT concentration is 1.3 μM, and Atto 647N labeled H2A-H2B concentration is ~650 nM. Two supershifted complexes (indicated by an asterisk) were enriched over M2 resin and analyzed by 5% PAGE, visualized by SYBR Gold or H4 (B) or H2B fluorescence (C). (D) is an overlay of 647 and 488 fluorescence.

2.4.5 H2A-H2B bound FACT interacts with tetrasomal H3-H4, and forms a supershifted complex.

Notably, only one supershifted (lower supershifted complex) is formed when the H2A-H2B concentration is low in the above assay (Figure 2.12). As H2A-H2B is increased, increasing amounts of the upper complex is formed (Figure 2.12 lane 6). Accordingly, we hypothesize that the upper supershifted complex contains more H2A-H2B dimers than the lower one. To test this idea, FACT was mixed with excess amount of H2A-H2B first, and then tetrasomes were added into the reaction mixture. Complexes were analyzed on a 5% PAGE, and visualized by SYBR Gold (DNA complexes) (Figure 2.14A). It was observed that two supershifted complexes are assembled, as described above. As predicted, the amount of lower supershifted complex is decreased, while the upper one is increased as H2A-H2B is titrated. As H2A-H2B concentration is high (240 nM), all the lower complexes are converted to the upper complexes (Figure 2.14A lane 4). It suggests that the upper supershifted complex contains more H2A-H2B dimers. Since FACT only binds a histone hexamer (one $(H3\text{-}H4)_2$ tetramer and one H2A-H2B dimer), the other complex may be formed due to the excess of H2A-H2B dimer binding to tetrasomal DNA.

To this end, tetrasome was reconstituted with a 79-bp DNA fragment, which does not contain ~36-bp free DNA at each side of $(H3\text{-}H4)_2$ tetramer but covers all protein-DNA binding sites on the $(H3\text{-}H4)_2$ tetramer. FACT-H2A-H2B and tetrasome, reconstituted on either 147-bp DNA or 79-bp DNA were combined and analyzed on a 5% PAGE. Bands were visualized by SYBR gold staining and fluorescence (H2B) (Figure 2.14B-C). We found that only one supershifted complex is assembled with a tetrasome of 79-bp DNA, while two complexes are formed with a tetrasome with a 147-bp DNA fragment as above

A


H2A-H2B
FACT
Tetrasome
+ +
+ +
M
bp
Supershifted comp.2
Supershifted comp.1
Nuc.
150
100
1 2 3 4
SYBR Gold Staining

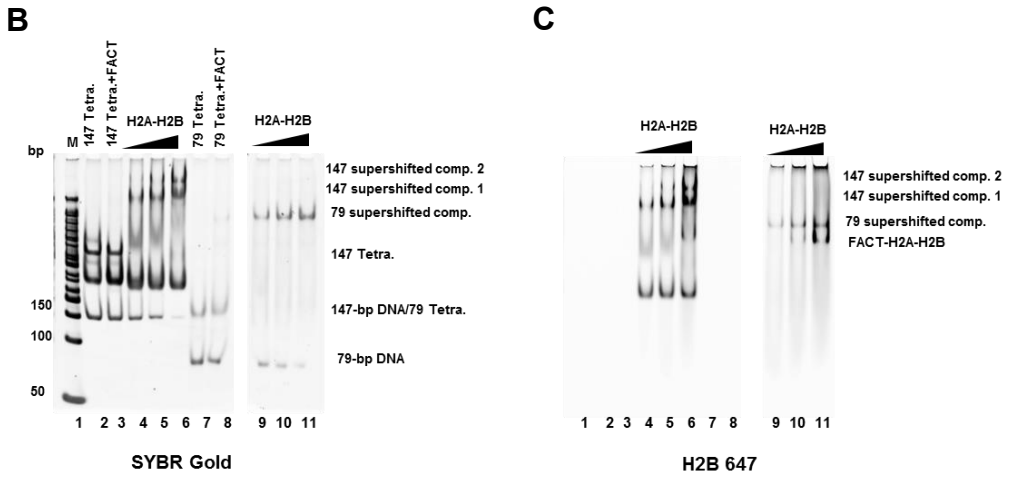


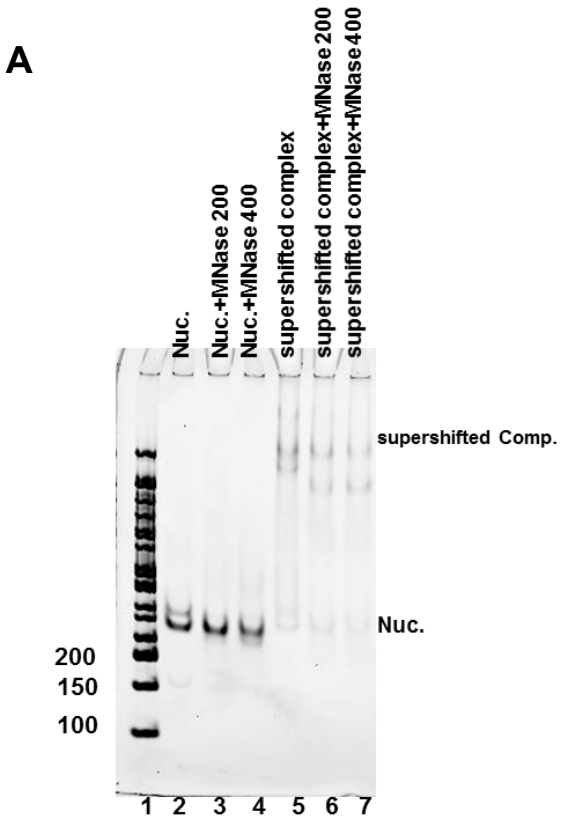
Figure 2.14. H2A-H2B bound FACT interacts with tetrasomal H3-H4, and forms a supershifted complex. (A) To determine if two supershifted complexes contain the same level of H2A-H2B, FACT was mixed with an increasing amount of H2A-H2B (160 nM and 240 nM) before adding tetrasome. FACT and tetrasome concentration were kept at 850 nM and 40 nM, respectively. Complexes were analyzed on a 5% PAGE, and visualized by SYBR Gold (A). As H2A-H2B is titrated, the intensity of the lower complex decreases, while the upper band increases. As H2A-H2B concentration is high (240 nM), all the lower complexes are converted to upper one. (B) and (C) In order to determine if free tetrasomal DNA is required for the supershifted complex, supershifted complexes were assembled by mixing tetrasomes reconstituted with either 79-bp or 147-bp DNA. Supershifted complex is also formed with tetrasome with 79-bp DNA. In addition, as H2A-H2B is titrated, supershifted complex 2 with147-bp DNA is assembled as expected, while only one complex is assembled for tetrasome with 79-bp DNA.

(Figure 2.14A). This confirms that FACT facilitates only one supershifted complex assembly, which contains one $(H3\text{-}H4)_2$ tetramer, one H2A-H2B dimer and DNA. This result also indicates that free DNA in the tetrasome is not required for the lower supershifted complex. It rules out the possibility that the supershifted complex is formed by the interaction of FACT-bound H2A-H2B with free tetrasomal DNA. Besides, this assay was performed at 150 mM NaCl, where histone H2A-H2B dimers and $(H3\text{-}H4)_2$ tetramers do not interact with each other. Thus, this indicates that the supershifted complex is not formed through FACT bound H2A-H2B interaction with tetrasomal H3-H4. Instead, this indicates that H2A-H2B bound FACT interacts with H3-H4 in the supershifted complex.

Together, our biochemical results show that two supershifted complexes contain different stoichiometries of H2A-H2B and H3-H4 histones. The upper complex may be comprised of two H2A-H2B dimers, one $(H3\text{-}H4)_2$ tetramer and DNA, while the lower one only has one H2A-H2B dimer, one $(H3\text{-}H4)_2$ tetramer and DNA. Additionally, we reveal that the supershifted complexes likely comprise interactions of H2A-H2B bound FACT with H3-H4 in the context of tetrasome.

2.4.6 DNA in the supershifted complex does not tightly wrap around histones.

To test if DNA in the supershifted complexes is tightly wrapped around histones, MNase digestion assay was applied. The supershifted complexes were assembled by histones and FACT with a 147-bp '601' DNA fragment. The complexes were enriched by using anti-FLAG affinity purification and subjected to MNase digestion, as described above. Digested products were analyzed on a 5% PAGE, and visualized by SYBR Gold staining (Figure. 2.15A). Protected DNA fragments were purified through MiniElute PCR


A
Nuc.
Nuc.+MNase 200
Nuc.+MNase 400
supershifted complex
supershifted complex+MNase 200
supershifted complex+MNase 400
supershifted Comp.
Nuc.
200
150
100
1 2 3 4 5 6 7

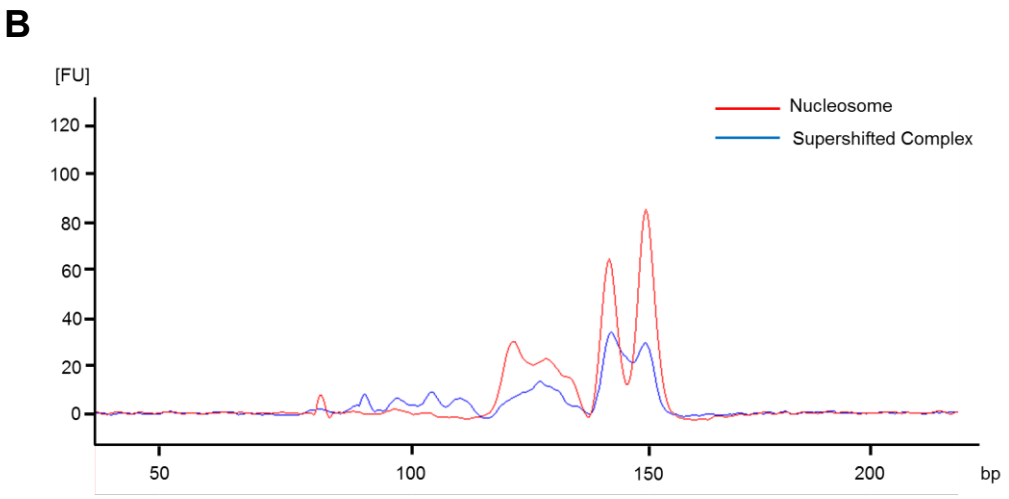


Figure 2.15. The supershifted complex is less resistant to MNase, compared to nucleosome reconstituted in vitro. Control nucleosomes and M2-purified supershifted complexes were mixed with different amount of MNase (100, 200 and 400 U), then analyzed on a 5% PAGE (A). Protected DNA fragments were analyzed by Bioanalyzer (B). It was shown that the nucleosome reconstituted by salt dialysis causes four significant pausing site for MNase (147, 140, 128 and 121-bp), while supershifted complexes have two main pausing sites for MNase (147 and 140-bp).

Table 2.2. FACT intermediate complex 1 with 147-bp DNA is less resistant to MNase, compared to nucleosome. The recovery rate is calculated as in Table 2.1. Only 21.7% of DNA was protected by 147-bp supershifted complexes, while 62.7% of DNA was protected by 147 NCP.

Size (bp)	Nucleosome (MNase 400U)	Supershifted complex (MNase 400U)
	Molarity (nmol/l)	Molarity (nmol/l)
121	11.6	0
128	12.3	0
140	15.3	10.9
147	19.8	7.3
Recovery Rate	**62.7%**	**21.7%**

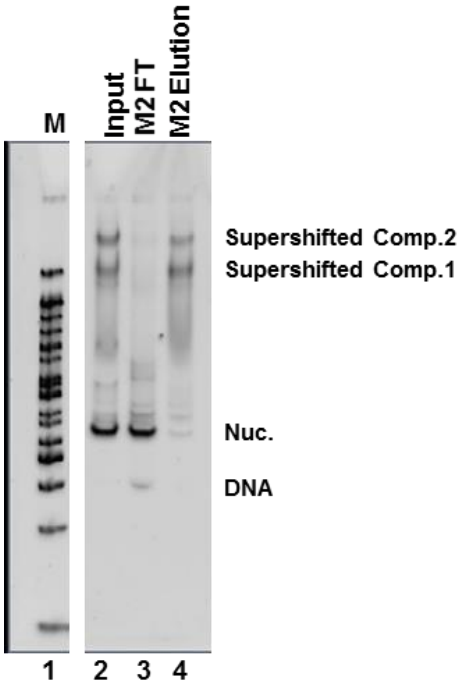
Purification Kit (QIAGEN) and analyzed by Bioanalyzer (Agilent) (Figure 2.15B). We found that nucleosomes reconstituted by salt dialysis exhibit three significant pausing sites for MNase (147, 140 and 121-bp), while supershifted complex with 147-bp DNA has two strong pause sites for MNase (147 and 140-bp) (Table 2.2). The MNase pause sites for the supershifted complex may be caused by nucleosome contamination as shown in Figure. 2.13. Regardless, the recovery rate of protected DNA is calculated. only ~22% for the supershifted complexes is protected, while the recovery rate for nucleosome is 63% (Table 2.2). This indicates that DNA in the supershifted complexes is likely less-stably organized than DNA in the nucleosome.

Our results suggest that FACT holds the partial components of nucleosome (a histone hexamer and DNA). Next, we asked if these supershifted complexes are homogenous in size. The supershifted complexes are assembled by 1.3 μM FACT, ~650 nM H2A-H2B and ~150 nM tetrasome with a 147-bp DNA fragment. The supershifted complexes were enriched through anti-FLAG tag affinity purification, and analyzed on a 5% PAGE. Bands were visualized by SYBR Gold staining (Figure 2.16A). Purified supershifted complexes were utilized for AUC. We found that two supershifted complexes are very homogenous with an $S_{(20,W)}$ value of about 18 (Figure 2.16B). Notably, two supershifted complexes are not distinguished in this assay.

2.4.7 FACT-H2A-H2B interaction and dimer-tetramer interaction are essential for the supershifted complex.

Having established that H2A-H2B facilitates FACT binding to tetrasomal H3-H4, we wanted to establish that interactions of H2A-H2B with FACT are crucial for the formation

A


M
Input
M2 FT
M2 Elution
Supershifted Comp.2
Supershifted Comp.1
Nuc.
DNA
1
2
3
4

B

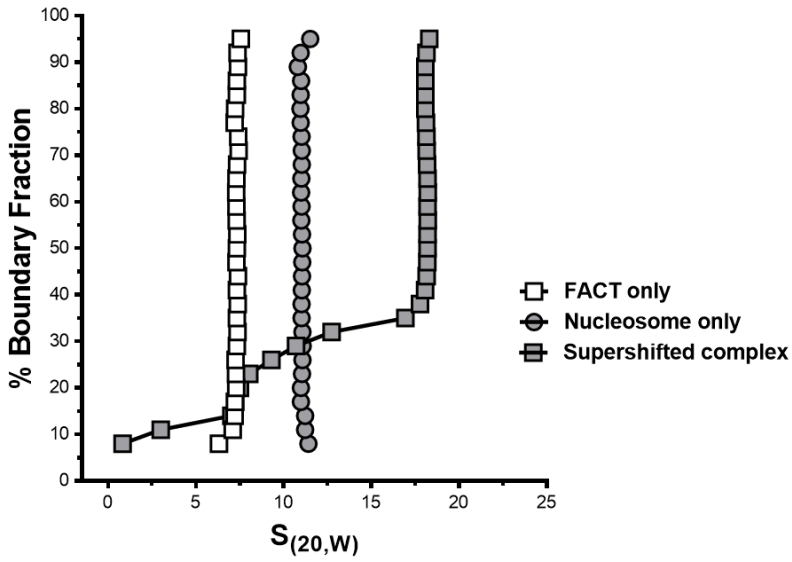


Figure 2.16. The supershifted complexes are homogenous in size. Tetrasome was kept at ~150 nM; FACT was 1.3 μM. Atto 647N labeled H2A-H2B concentration was ~650 nM. FACT was mixed with H2A-H2B before adding tetrasome. Supershifted complexes were purified via M2 resin and analyzed on a 5% PAGE gel, visualized by SYBR Gold (A). Purified supershifted complexes were analyzed by (B).

of the supershifted complex. To this end, FACT ΔCTD (Spt16 Δ933-1047 a.a.), previously shown to be deficient for H2A-H2B binding, was expressed and purified as previously described (Winkler et al. 2011). AUC-FDS confirmed that FACT ΔCTD does

not bind to H2A-H2B, since no change in S-value was observed upon mixing 100 nM Alexa 488 labeled H2A-H2B with 200 nM FACT ΔCTD (Figure 2.17). It further confirms that hSpt16-CTD is the only binding site for H2A-H2B, while hSSRP1 does not bind H2A-H2B.

We next wanted to test whether FACT Δ CTD is capable of forming a supershifted complex. Complexes were assembled as above and analyzed on a 5% PAGE, visualized by SYBR Gold (DNA complexes), fluorescence (H4) and fluorescence (H2B) (Figure 2.18). FACT ΔCTD does not form any complex with histone and DNA. This suggests that the ability of FACT to interact with H2A-H2B is essential for complex formation, in agreement with published work (Tsunaka et al. 2016).

Next, we asked whether H2A-H2B and H3-H4 interaction is required for these supershifted complexes. To this end, we employed histone mutations in H3I51 (A) and H4Y98 (H). In the context of a nucleosome, H4Y98 forms a hydrophobic contact with the docking domain of H2B near its base, while H3I51 stabilizes the very end of the H2A docking domain near the DNA exit region (Figure 2.19A). Of the two, H4Y98H has more severe effects on the stability of dimer-tetramer interaction (Hsieh et al. 2013, Ramachandran et al. 2011). Point of mutation of H4Y98 is lethal (Santisteban et al. 1997). According to the crystal structure of the Spt16MD-AID with the $(H3\text{-}H4)_2$ tetramer, H3I51 and H4Y98H are not involved in FACT-$(H3\text{-}H4)_2$ interaction (Tsunaka et al. 2016). We

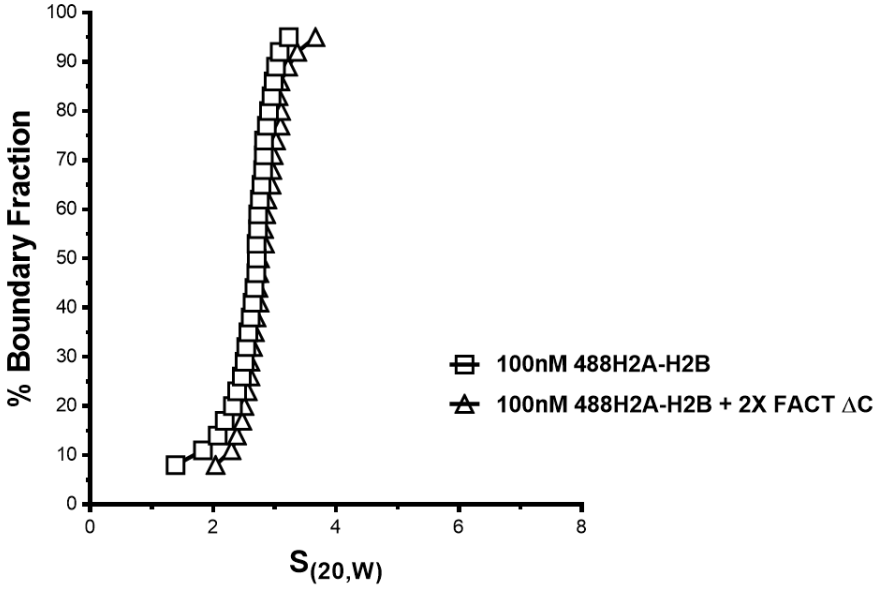


Figure 2.17. FACT ΔC does not bind to H2A-H2B. 100 nM Alexa 488 labeled H2A-H2B was mixed with 200 nM FACT ΔC, and samples were subjected to AUC-FDS. The $S_{(20,W)}$ value of H2A-H2B alone is ~2.5. Addition of FACT ΔC does not alter the $S_{(20,W)}$ value of H2A-H2B, suggesting that FACT ΔC does not bind H2A-H2B dimer.

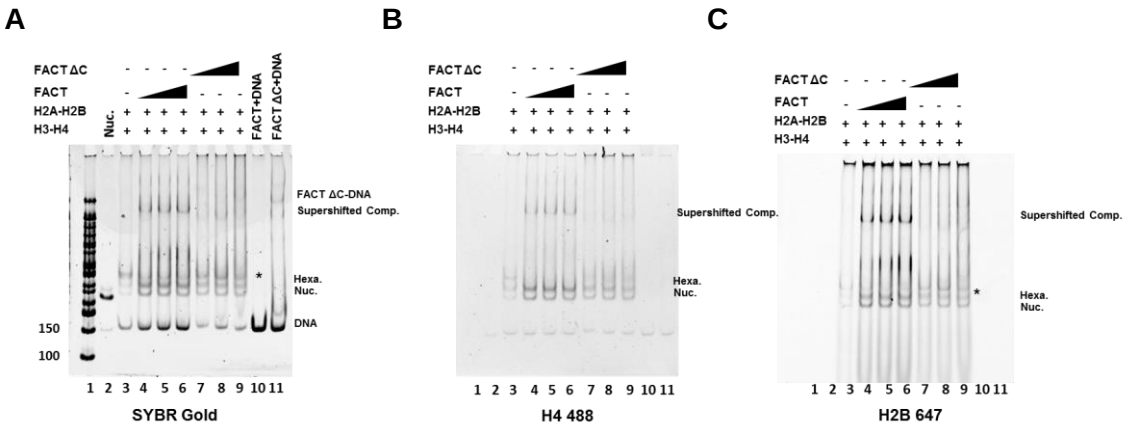
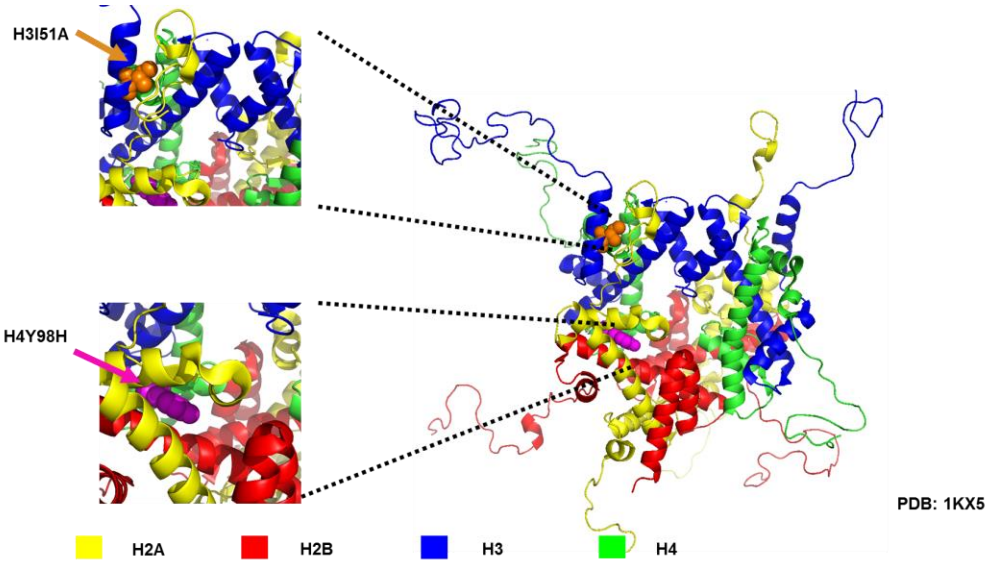


Figure 2.18. FACT ΔC does not facilitate nucleosome and supershifted complexes formation. 50 nM H3-H4 and 100 nM H2A-H2B mixed with FACT or FACT ΔC. 25 nM DNA was added, and analyzed on a 5% PAGE, visualized by SYBR Gold, or by fluorescence as indicated. FACT ΔC does not facilitate assembly of the supershifted complexes, nor does it facilitate nucleosome assembly. FACT ΔC does not prevent nonspecific binding between H2A-H2B and DNA (asterisk).

A



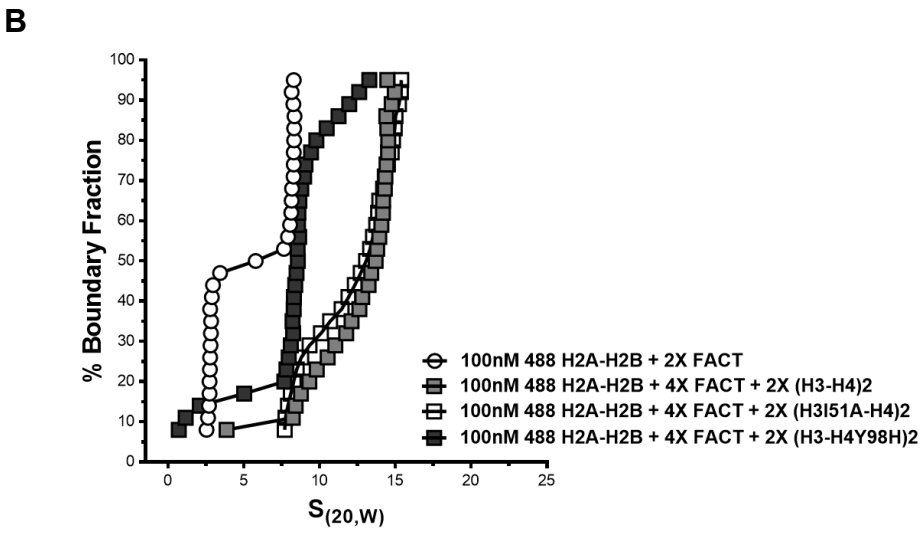


Figure 2.19. H3I51A does not affect the formation of FACT-histone hexamer complex, while H4Y98H is incapable of complex formation. (A) The position of H3I51 and H4Y98 in the context of histone octamer are indicated in orange and magentas, respectively. (B) 100 nM Alexa 488 labeled H2A-H2B was mixed with 200 nM H3-H4, H3I51A-H4 or H3-H4Y98H, and subjected to AUC-FDS. H3I51A does not affect FACT-histone hexamer complex formation, while H4Y98H abolishes the formation of FACT-histone hexamer complex.

first asked if these histone mutants affect FACT interaction with a histone hexamer in absence of DNA. AUC-FDS (using fluorescently labeled H2A-H2B) was performed as above. We found that H3I51A does not significantly affect FACT interaction with a histone hexamer, while H4Y98H abolishes the formation of FACT-histone hexamer complexes (Figure 2.19B). This indicates that the interaction between H2B and H4 is essential for FACT holding a histone hexamer. In other words, H2A-H2B interacts with H3-H4 in the FACT-histone hexamer complex. Since H2A-H2B dimers and $(H3\text{-}H4)_2$ tetramers do not interact with each other at 150 mM NaCl, this suggests that FACT promotes H2A-H2B and H3-H4 interaction in a ternary complex. Next, we tested if the H2A-H2B and H3-H4 interaction is required for the formation of supershifted complex. The supershifted complex was assembled from wild type or mutant histones. The supershifted complexes were analyzed on a 5% PAGE, and visualized by SYBR gold staining (Figure 2.20). It was observed that both H3I51A and H4Y98H impedes the formation of supershifted complexes (Figure 2.20 lane 3 and 4). Thus, H2A-H2B and H3-H4 interaction are also required for these supershifted complexes. Surprisingly, we found that H3I51A does not significantly affect FACT binding histone hexamer, whereas it affects the supershifted complexes assembly. To sum up, we show that FACT binding H2A-H2B and (H2A-H2B)-(H3-H4) interaction are required for the supershifted complex. Additionally, FACT promotes (H2A-H2B)-(H3- H4) interactions in order to hold a histone hexamer.

2.4.8 FACT has only moderate effects on gene transcription.

Having shown that FACT forms a homogenous complex with histones and DNA, we subsequently determined the role of FACT in regulating gene transcription elongation by using a simplified in vitro transcription system (Kuryan et al. 2012). In previously published

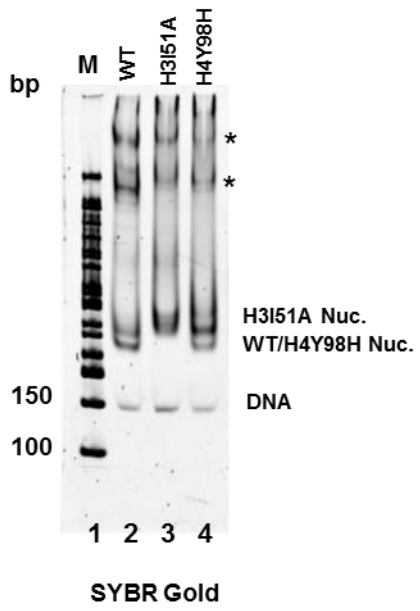
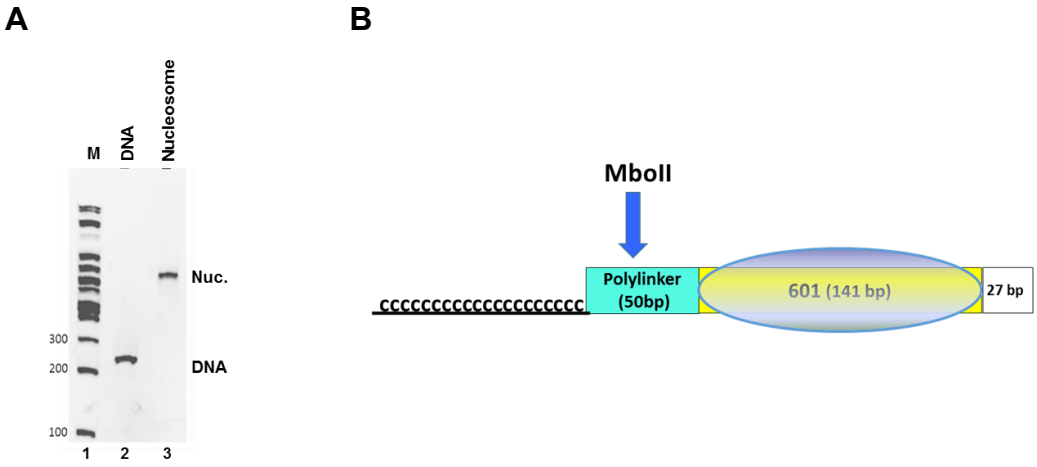


Figure 2.20 H2A-H2B interacts with H3-H4 in the supershifted complexes. 147-bp '601' DNA concentration was kept at 25 nM. H3-H4, H3I51A-H4 or H3-H4Y98H tetramer and H2A-H2B dimer concentration were kept at 50 nM and 100 nM, respectively. 400 nM FACT was incubated with histones at RT for 10 mins, and then DNA was added. The reaction mixture was incubated at RT for another 30 mins. The supershifted complexes were analyzed on a 5% PAGE, and visualized by SYBR Gold. H3I51A or H4Y98H negatively affects the formation of supershifted complexes (asterisk).

work, it has been shown that FACT does not facilitate gene transcription (Kuryan et al. 2012). Here, we wanted to see if FACT affects RNA pol II pausing sites.

To this end, the 147-bp '601' DNA were flanked by 100-bp polylinker DNA sequences and 27-bp DNA sequences (Figure 2.21B). Mononucleosome was reconstituted with *Xenopus laevis* histones using salt dialysis (Figure 2.21A). Nucleosome concentration was kept at 5 nM, and FACT was titrated. Upon addition of NTPs and RNase H, the chromatin template was transcribed by RNA pol II. The RNA transcripts were analyzed on a 6.5% acrylamide sequencing gel (Figure 2.21C). FACT on its own only has moderate effects on gene transcription, whereas FACT stimulates transcription in presence of the ATP-dependent chromatin remodeler RSC. Notably, two RNA pol II pausing sites were observed in the presence of FACT, which are caused by H2A-H2B-DNA (~86-bp) and H3-H4-DNA interaction (~105-bp). Since FACT does not remove these pausing sites, FACT does not remove H2A-H2B and H3-H4 from nucleosomes. The amount of transcript indicative of these two pause sites are increased along with increased full length transcripts. This implies that histones remain at the same position after the passage of RNA pol II. We speculate that FACT facilitates RNA pol II progression through nucleosomes by transiently breaking the DNA-histone interactions. It was suggested that strong histone-DNA interactions, such as H3-H4-DNA around nucleosome dyad and two H2A-H2B-DNA interactions at the edge of nucleosome, affect RNA pol II passage through nucleosome (Hall et al. 2009). No RNA pol II pausing sites are observed around nucleosome dyad and promoter-distal dimer-DNA interaction regions. This indicates that FACT may disrupt these histone-DNA interactions, and thus stimulating full length transcripts. Notably, as FACT concentration is increased, the full length transcription


A
M
DNA
Nucleosome
Nuc.
DNA
300
200
100
1
2
3
B
MboII
Polylinker
(50bp)
601 (141 bp)
27 bp
ccccccccccccccccccc

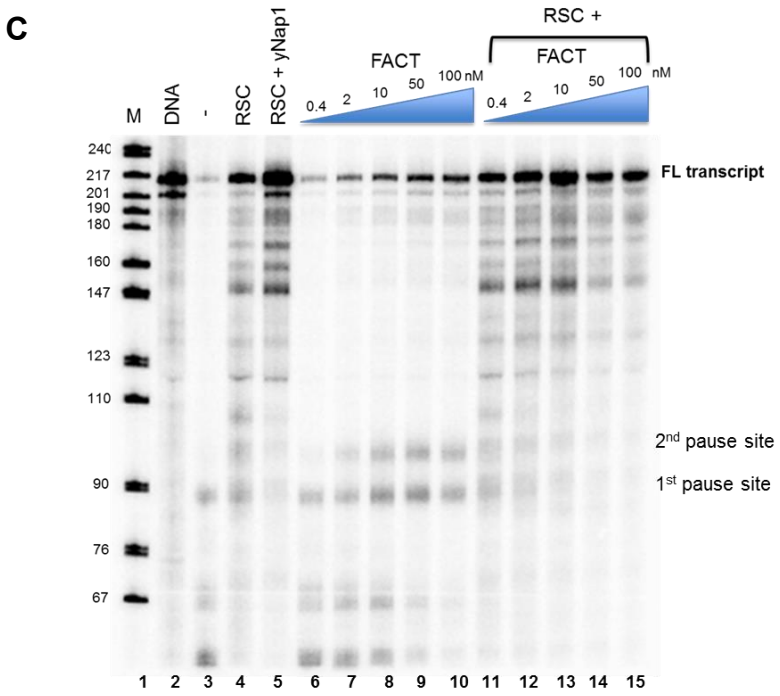


Figure 2.21. **The role of FACT in transcription elongation**. A) Nucleosomes were reconstituted on the transcription template (B), and analyzed by native PAGE. C) The transcription output is analyzed on a 6.5% acrylamide sequencing gel. In the absence of RSC, two transcription pause sites are observed. The 1st pause site is due to the 1st dimer at front of RNA pol II. The 2nd pause site is specific for FACT. In the presence of RSC and FACT, all pause sites are removed. FACT *per se* slightly stimulates gene transcription. In the presence of RSC, the amount of full length transcript increases, but decreases at higher FACT concentration. Compared to FACT with RSC, Nap1 with RSC results in more transcripts. This experiment was performed by H. Scherman at the Protein Expression/Purification Facility at CSU.

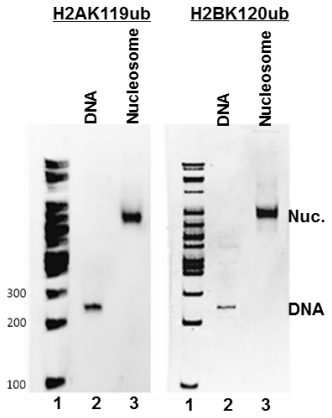
levels do not continually increase, which is in the line with previous results (Kuryan et al. 2012, Orphanides et al. 1999).

Next, we set out to investigate the role of histone ubiquitination in FACT function during transcription elongation by using the same transcription system. Mononucleosomes were reconstituted onto the transcription template as described above, using octamers containing either H2AK119ub or H2BK120ub (Figure 2.22A), and the transcription reaction was performed as described above. We notice that the results are similar to those obtained for unmodified nucleosomes in Figure 2.21C. In contrast, either H2AK119ub or H2BK120ub facilitates FACT activity in transcription elongation in the absence of RSC (Figure 2.22B). We noticed that nucleosomes with H2AK119ub have slightly increased amounts of transcripts than nucleosome with H2BK120ub. In the presence of RSC, the similar level of full length transcript is obtained for WT, H2AK119ub and H2BK120ub nucleosome. It indicates that histone ubiquitination per se does not affect FACT activity in gene transcription. However, this experiment was done only once, and will be repeated.

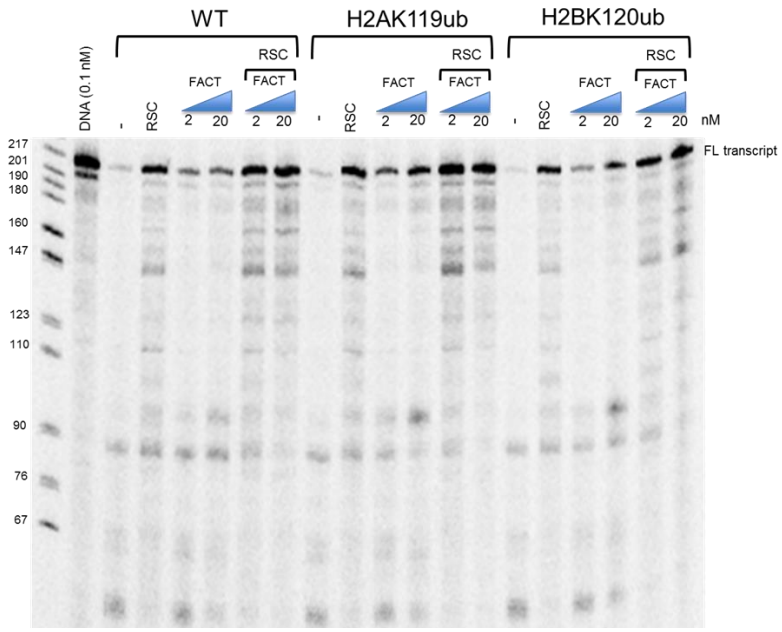
2.5 Discussion

FACT plays important roles in gene transcription, DNA replication and DNA repair (Keller and Lu 2002, Schlesinger and Formosa 2000, Wittmeyer and Formosa 1997). Our in vitro results provide mechanistic insight into how hFACT regulates nucleosome structure. We found that FACT, in absence of other factors, does not bind to mononucleosome or disassemble nucleosomes. This is likely due to the fact that its binding sites on histones are buried by either nucleosomal DNA or other histones in the context of the nucleosome

A


H2AK119ub
H2BK120ub
DNA
Nucleosome
DNA
Nucleosome
Nuc.
DNA
300
200
100
1 2 3
1 2 3

B


WT
H2AK119ub
H2BK120ub
DNA (0.1 nM)
RSC
FACT
2 20
nM
217
201
190
180
160
147
123
110
90
76
67
FL transcript

C

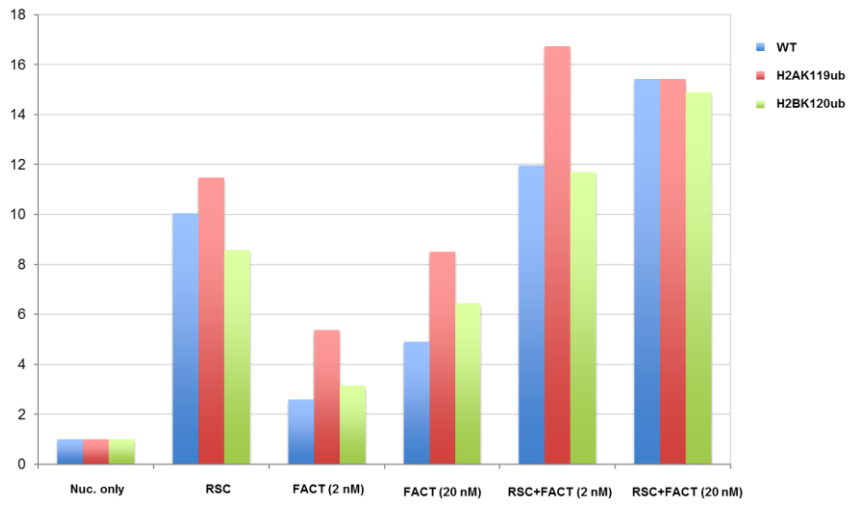


Figure 2.22. **The role of histone ubiquitination in FACT function during transcription elongation.** A) Nucleosomes were reconstituted with *Homo sapiens* ubiquitinated histones. Nucleosomes were analyzed on a 5% PAGE gel. B) The transcription output was analyzed on a 6.5% acrylamide sequencing gel. In the absence of RSC, two transcription pause sites were observed as in Fig. 2.21. C) Full length transcription products in (B) were quantified. In the absence of RSC, both H2AK119ub and H2BK120ub slightly stimulate FACT-dependent generation of full length transcript. In the presence of RSC, H2AK119ub and H2BK120ub do not affect FACT function. Similar level of full length transcript is obtained for WT, H2AK119ub and H2BK120ub nucleosomes. This experiment was also performed by H. Scherman at the Protein Expression/Purification Facility at CSU.

(Tsunaka et al. 2016). Thus, FACT does not initialize nucleosome disassembly on its own. Instead, our data suggest that FACT tethers partial components of nucleosome, and thus forming an unstable complex. In addition, we show that FACT is a nucleosome assembly factor. The role of histone ubiquitination in FACT-(H2A-H2B) interaction, FACT assembly activity, and gene transcription was also investigated.

FACT holds one $(H3\text{-}H4)_2$ tetramer and one H2A-H2B dimer simultaneously.

Here, we show that FACT binds a histone hexamer, composed of one $(H3\text{-}H4)_2$ tetramer and one H2A-H2B dimer. In addition, we found that H4Y98H, through disrupting H2B-H4 interaction, impedes FACT binding a histone hexamer. This indicates that the interaction between H2A-H2B and H3-H4 (presumably similar to what is observed in the nucleosome) is required for FACT binding all histones together. Since histones alone do not interact with each other at physiological ionic strength, FACT facilitates H2A-H2B interaction with H3-H4. How does FACT promote H2A-H2B and H3-H4 interaction? We propose that FACT binds the H2B N-terminal tail via Spt16-CTD as shown in previous work (Tsunaka et al. 2016), and then folds over at the top of H2B-H4 interface to interact with the distal H2A docking site at the surface of (H3-H3'-H4') and the proximal H4 L1 loop (Figure 2.23). In this way, FACT might be able to tie up H2A-H2B and H3-H4, and stabilize H2A-H2B and H3-H4 interactions. In other words, FACT works as 'DNA' and holds both H2A-H2B and H3-H4 to form a compact complex. Sedimentation coefficient is positively related with molecular weight and negatively related to frictional coefficient, this model also explains why FACT-histone hexamer complex has much larger S-value than FACT-H2A-H2B.

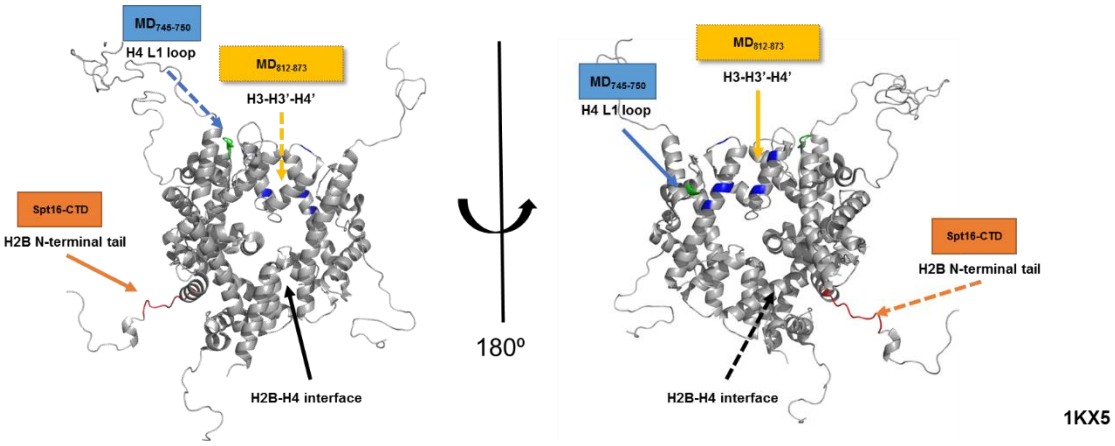


Figure 2.23. A model for FACT interaction with a histone hexamer. For structural review, Spt16-CTD binds H2B N-terminal tail. Spt16-$MD_{812-873}$ binds the H2A docking site at the surface of H3-H3'-H4', while Spt16-$MD_{745-750}$ interacts with H4 L1 loop. Based on our AUC data, we propose that Spt16-CTD binds H2B first, and then FACT folds over at the top of H2B-H4 interface interacting with H3-H3'-H4' and H4 L1 loop. FACT works as 'cane' to lock H2A-H2B with H3-H4.

Mechanistic insight into how FACT enhances the global accessibility of nucleosomal DNA

At lower transcription rates, hexasomes or tetrasomes could survive after passage of RNA pol II (Dion et al. 2007, Jamai et al. 2007, Thiriet and Hayes 2005). Tetrasome only could cause strong pause sites for RNA pol II (Bondarenko et al. 2006). How does RNA pol II overcome this obstacle without depletion of H3-H4? Likely, H3-H4-DNA interactions need to be transiently disrupted and re-formed during gene transcription. Here, we have shown that FACT tethers a histone hexamer to form a homogenous supershifted complex with DNA. In this way, DNA is less-stably organized compared to nucleosomal DNA. This may due to the fact that FACT-(H2A-H2B) and FACT-(H3-H4) interaction is at least partly incompatible with nucleosomal DNA-histone interaction. It is consistent with the idea that FACT 'reorganizes nucleosomes' (Xin et al. 2009). In agreement, hFACT holds partial components of nucleosome as a result of FACT removing H2A-H2B from nucleosome, which was reconstituted with two DNA fragments (33/112-bp) (Tsunaka et al. 2016). Similarly, yFACT enhances restriction enzyme digestion rate for nucleosomal DNA (Xin et al. 2009). Our results provide mechanistic insight into how FACT reorganizes nucleosome via tethering partial nucleosome components, which in turn suggests how FACT facilitates gene transcription. Indeed, FACT stimulates gene transcription in an in vitro transcription system. No pausing sites are caused by H3-H4-DNA interaction around nucleosome dyad, which was identified as the strongest histone-DNA interaction in nucleosome (Hall et al. 2009). This is consistent with our idea that FACT is able to enhance the global accessibility of nucleosomal DNA.

FACT-(H2A-H2B) and (H2A-H2B)-(H3-H4) interaction are essential for the formation of the ternary complex. On the other hand, we show that FACT binding H2A-H2B forms a supershifted complex with a tetrasome with 79-bp DNA. It suggests that free DNA in tetrasome is not required for the supershifted complex. H2A-H2B does not interact with H3-H4 in absence of DNA or histone chaperones at 150 mM NaCl. Thus, FACT also interacts with H3-H4 in the supershifted complex. Together, this suggests that interaction with H2A-H2B facilitates FACT binding H3-H4 in the context of tetrasome. Subsequently, FACT promotes H2A-H2B and H3-H4 interaction, and in turn facilitates the recruitment of H2A-H2B to tetrasome, resulting in the assembly of the supershifted complex.

FACT maintains chromatin integrity

We report that FACT also has nucleosome assembly activity in vitro, through facilitating tetrasome assembly, as well as H2A-H2B deposition onto tetrasomes and hexasomes. This is consistent with the finding that in yeast, histone turnover rates are enhanced as FACT activity is compromised (Jamai et al. 2009). How does FACT facilitate H2A-H2B deposition onto tetrasome? Likely, FACT facilitates H2A-H2B deposition by assembling the supershifted complex, where FACT promotes H2A-H2B interaction with tetrasomal H3-H4. Alternatively, FACT may facilitate H2A-H2B deposition by preventing nonspecific H2A-H2B-DNA interaction.

Additionally, we also show that Spt16-CTD is essential for FACT tethering nucleosome components. The contribution of other functional domains of FACT in FACT assembly/disassembly activity is still unknown. For example, the role of HMG domain in SSRP1 may affect FACT activity. Phosphorylated FACT was used in this study, which

prevents FACT-DNA binding. However, it is possible that the DNA binding property of FACT could facilitate its assembly activity.

We determined the role of histone ubiquitination in FACT assembly activity. FRET-based competition assay was performed to test if mono-ubiquitination disrupts FACT-(H2A-H2B). Since H2AK119ub and H2BK120ub are not located at the known interface of FACT-(H2A-H2B) (Kemble et al. 2015), we found that mono-ubiquitination does not affect FACT-(H2A-H2B) interaction as expected. Subsequently, we asked if mono-ubiquitination affects FACT-mediated nucleosome assembly activity. Our data suggest that mono-ubiquitination at either H2AK119 or H2BK120 does not alter FACT assembly activity. Also, H2AK119ub and H2BK120ub do not affect FACT function in gene transcription. However, it has been shown hFACT more efficiently stimulates gene transcription in the presence of H2BK120ub in vitro transcription assay (Pavri et al. 2006). The discrepancy may be caused by different experimental designs. In the previous work, the contribution of H2BK120ub in FACT activity during transcription elongation was determined in the presence of several transcription factors, such as p300, PARP-1 and mediator. Likely, H2BK120ub with help of other transcription factors regulates FACT function.

How is FACT function in nucleosome structure regulated?

Our data suggest that FACT not only assembles nucleosome, but enhances the accessibility of nucleosomal DNA. It indicates that FACT regulates nucleosome structure through multiple mechanisms (Figure 2.24). However, it is still unclear how FACT activity is regulated. We speculate that FACT activity is regulated by histone turnover rate. As H2A-H2B dynamics is low, FACT mainly facilitates H2A-H2B dimer deposition onto tetrasome

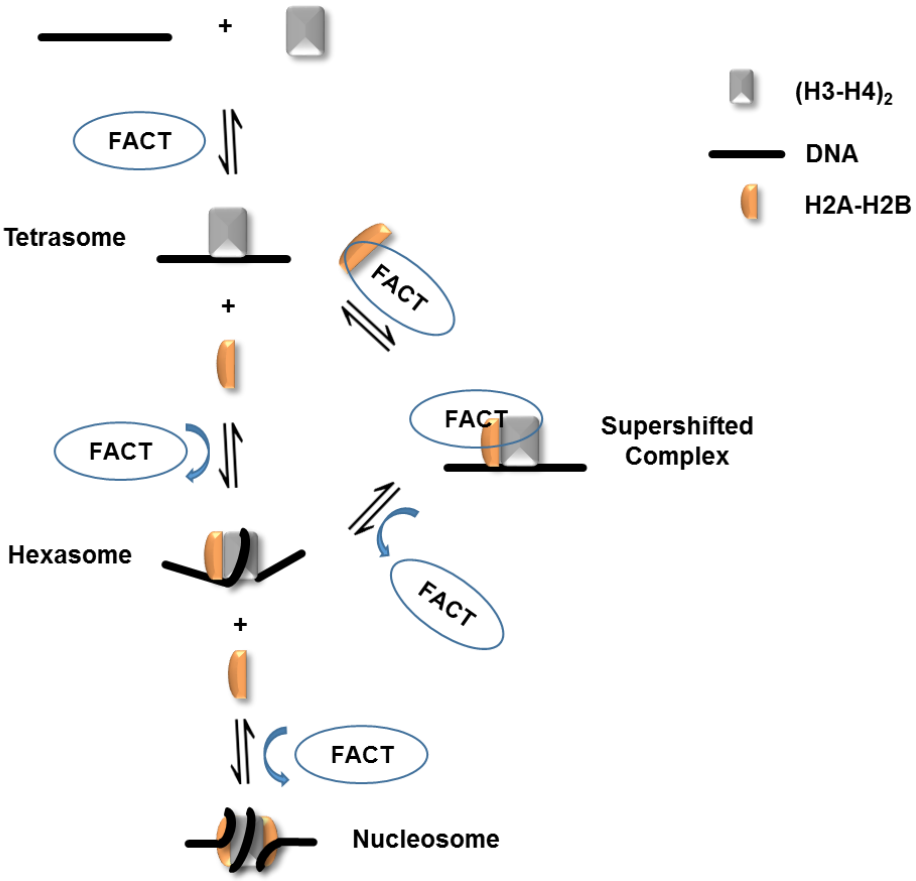


Figure 2.24. FACT regulates nucleosome structure via multiple mechanisms. 1) FACT facilitates tetrasome assembly. As H2A-H2B concentration is low, FACT facilitates H2A-H2B deposition onto tetrasome and hexasome, thereby facilitating nucleosome assembly. 2) As H2A-H2B concentration is high, FACT-H2A-H2B complex is enriched. H2A-H2B bound FACT interacts with tetrasomal H3-H4, and thus forming a supershifted complex. In the way, FACT is able to enhance nucleosomal DNA accessibility, which may facilitate transcription through chromatin. Hexasome may be formed, as FACT disassociates from the supershifted complex.

and hexasome, and thus facilitating nucleosome assembly, dictating its nucleosome assembly function. As H2A-H2B dynamics is high, FACT-H2A-H2B complexes are enriched, which results in the formation of the supershifted complex with tetrasome. Hexasome may be assembled as FACT disassociated from the supershifted complex.

FACT function may be also regulated by post translational modifications. It has been shown that FACT interacts with PARP-1, and activation of PARP-1 results in the poly(ADP-ribosyl)ation of hFACT (Huang et al. 2006). Given the highly charged nature of the modification, poly(ADP-ribosyl)ation may increase FACT-histone interaction, which may affect FACT assembly activity. On the other hand, FACT function may be also regulated by histone PTMs. It has been shown that histone PTMs have effects on the recruitment of FACT (Carvalho et al. 2013). Histone PTMs may also affect FACT interaction with histones, and results in altering FACT assembly function. Additionally, histone PTMs also have an effect on nucleosome structure. Histone PTMs destabilizing nucleosome may facilitate FACT recruitment to nucleosomes. In sum, many factors have potential roles in the regulation of FACT activity. FACT is a chromatin regulator with distinct role in chromatin structure under different DNA-dependent cellular processes.

2.6 Acknowledgement

We thank Daniel Krzizike for doing all the AUC-FDS experiments and analyzing the data. We also than Dr. Hataichanok Scherman for doing the in vitro transcription assay and providing histones used in this project. Meanwhile, we would like to thank Dr. Tingting Yao for preparing H2AK119ub-H2B and H2A-H2BK120ub dimer. We thank Dr. Serge Bergeron for the valuable suggestions regarding the nucleosome assembly assay.

CHAPTER 3

THE EFFECTS OF HISTONE MODIFICATION ON NUCLEOSOME STRUCTURE

(RESULTS RELATED TO H3Y41E AND H3T45E HAVE BEEN PUBLISHED)

Brehove, M., T. Wang (Joint author), J. North, Y. Luo, S. J. Dreher, J. C. Shimko, J. J. Ottesen, K. Luger and M. G. Poirier (2015). "Histone Core Phosphorylation Regulates DNA Accessibility." J Biol Chem.

3.1 Summary

Histone posttranslational modifications (PTMs) have been described in the last century. Increasing numbers of histone PTMs have been identified, such as acetylation (ac), methylation (me), phosphorylation (phos) and ubiquitination (ub). Decades ago, most histone PTMs were identified in the core histone tails. Histone PTMs affect chromatin structure by multiple mechanisms. It was proposed that serving as a binding site for regulatory proteins is the major potential roles for histone PTMs from one or more histone tails, namely 'histone code'. Alternatively, histone PTMs may directly regulate chromatin structure. These histone modification sites are likely located in the buried regions of nucleosomes. Highlighted in this study is how select histone PTMs directly affect chromatin structure.

Histone PTMs selected for this project are located in the histone structural domains, such as H3Y41phos, H3T45phos, H3R42me2 and H2BK34ub. These modifications have no effects on histone octamer refolding and nucleosome reconstitution. By doing Small Angle X-ray Scattering (SAXS) and restriction enzyme digestion assay, we were able to show that histone PTM mimics occurring at DNA entry-exit region of nucleosome facilitate 'DNA

breathing'. Additionally, our preliminary data suggest that ubiquitination at H2BK34 does not affect histone-DNA interaction.

3.2 Introduction

An increasing numbers of histone PTMs have been well studied. Various histone PTMs are associated with the regulation of many DNA dependent processes, such as gene transcription and replication (Chen et al. 2008, Strahl and Allis 2000, Williams et al. 2008). It has been proposed that histone PTMs affect chromatin structure by direct or indirect mechanisms (Cosgrove et al. 2004). Histone PTMs may weaken the interactions between DNA and histone, histone and histone, or histone and DNA-histone complexes. Alternatively, modifications may serve as a binding sites for those ATP-dependent chromatin remodelers or histone chaperones that contain chromo- or bromo-domain to "read" these marks.

Histone PTMs occurring at distinct nucleosome structural regions have different potential effects on nucleosome stability (Manohar et al. 2009). The distinct regions of nucleosomes were defined as described (Luger et al. 1997). The entry-exit regions of nucleosomal DNA is organized by parts of the N-terminal tail of H3, which interacts with nucleosomal DNA, and throughout the first alpha helix of H3. The dyad region of nucleosomes refers to the central axis of symmetry through the nucleosome. It was proposed that histone PTMs throughout the entry and exit regions of nucleosomes facilitate DNA unwrapping from histone octamers, and that histone PTMs around the nucleosome dyad facilitate nucleosome disassembly (Manohar et al. 2009). This idea was supported by a large body of experimental evidence.

Here, our study is focused on how PTMs in the histone core regions affect nucleosome structure. Histone PTMs selected for this purpose are H3Y41phos, H3R42me2, H3T45phos and H2BK34ub (Figure 3.1A). These modifications are selected based on their structural and biological function importance. H3Y41, H3R42 and H3T45 are located at H3 αN helix, while H2BK34 is located at H2B N-terminal tail (Figure 3.1 B-E). Residues in H3 αN helix make important interactions with nucleosomal DNA. It has been shown that mutations at H3 αN helix significantly increases nucleosome sliding (Ferreira et al. 2007). Thus, we propose that H3Y41phos, H3R42me2 and H3T45phos facilitate nucleosomal DNA breathing.

H3Y41 phosphorylation was identified in human leukemic cells, and is added by the Janus kinase 2 (JAK2) (Dawson et al. 2009). It has been shown that H3Y41phos has a potential role in gene transcription (Dawson et al. 2012). Dawson and colleagues showed that H3Y41phos prevents heterochromatin protein 1α from binding to chromatin (Dawson et al. 2009), and it was suggested that H3Y41phos prevents heterochromatin formation. On the other hand, H3Y41phos may facilitate chromatin decompaction. All these evidence suggests that H3Y41phos plays an important role in DNA dependent processes. From a structural review, H3Y41 is positioned at the N-terminal tail of H3 and points into the minor groove of the last turn of nucleosomal DNA (Figure 3.1B). Histone PTMs occurring at this position may affect DNA wrapping around histones. Specially, phosphorylation occurring at this residue will introduce a negative charge to its side chain, which may disrupt histone-DNA interaction.

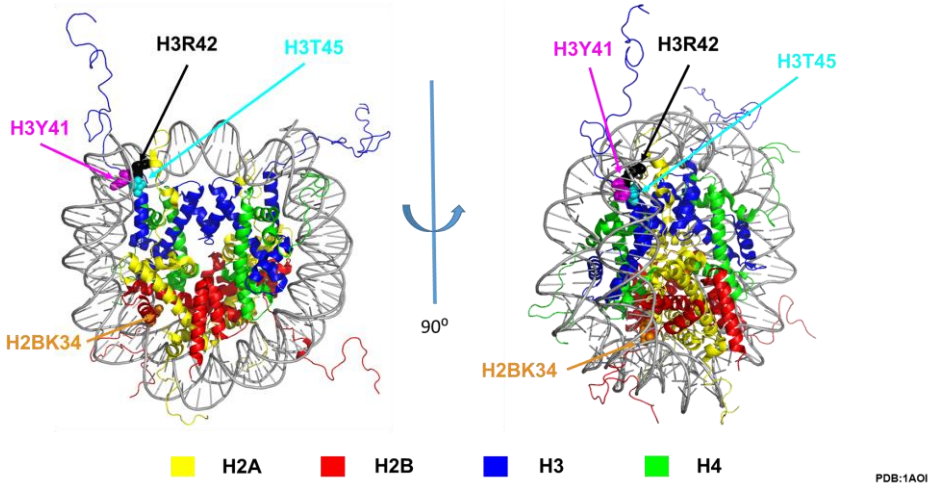
H3R42 is a lysine in yeast, but it is arginine in most higher eukaryotes. H3R42, located at the N-terminal tail of H3, is also involved in histone-DNA interaction (Figure 3.1C). Some

evidence showed that point mutation of this residue to alanine in yeast can cause cells to grow slowly and alter gene transcription (Hyland et al. 2011). Besides, H3R42 is methylated by CARM1 (coactivator-associated arginine methyltransferase 1). CARM1 binds to P160 coactivators and facilitates the transcription by nuclear receptors (Lee et al. 2005). Recently, it has been reported that H3R42me2 facilitates gene transcription in an in vitro transcription system (Casadio et al. 2013). Although its mechanism still needs to be elucidated, it was speculated that H3R42me2 disturbs the interactions between DNA and the histone octamer.

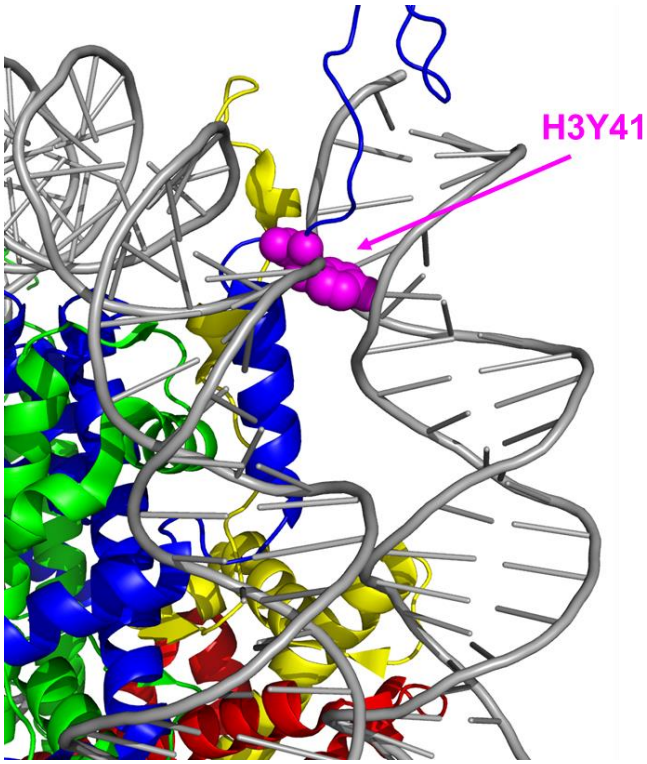
H3T45 is phosphorylated by S phase regulatory kinase Cdc7-Dbdf4 (Baker et al. 2010). H3T45phos is associated with apoptosis. H3T45phos is also linked to DNA replication. Depletion of this modification causes replication defects (Baker et al. 2010). Again, due to the lack of biochemical experimental data, the mechanism of how H3T45phos regulates nucleosome structure is still unclear. H3T45 is located at the H3 αN helix, which points directly at the DNA phosphate backbone, which organizes the last turn of DNA (Figure 3.1D). Compared to H3Y41phos, H3T45phos may have similar effects on DNA-histone interaction.

H2BK34 ubiquitination was modified by Male Specific Lethal 1 (MSL1), together with MSL2 (Wu et al. 2011). This modification directly regulates gene transcription by facilitating RNA pol II processivity (Wu et al. 2014). From structural review, H2BK34 is located in the H2B N-terminal tail that emerges from two gyres of the nucleosomal DNA superhelix (Figure 3.1E). Since H2BK34 is buried by nucleosomal DNA, H2BK34 may be not accessible in the context of nucleosome. However, it has been shown that this residue can be modified in the context of nucleosome (Wu et al. 2011). Since H2A-H2B-

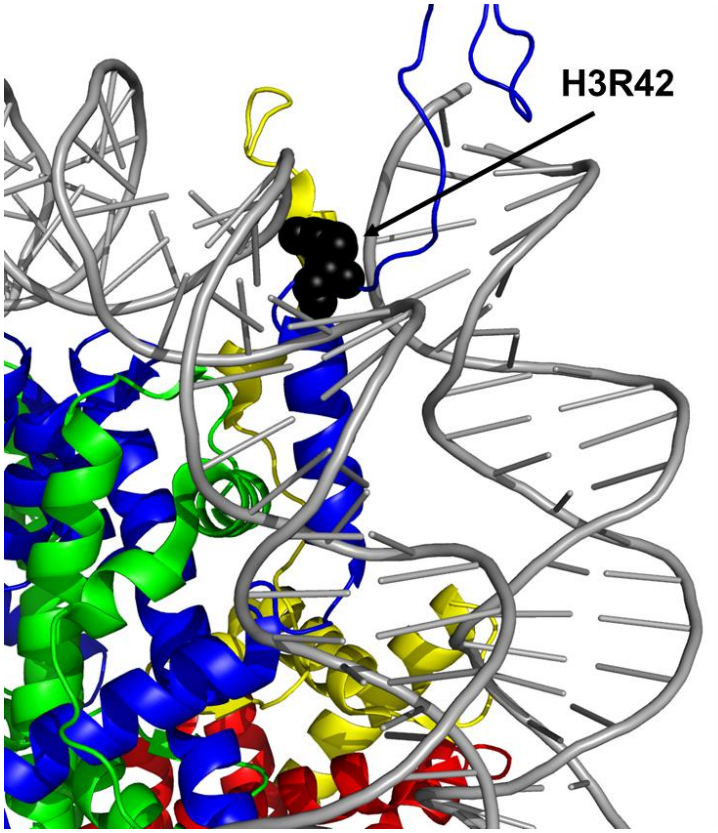
A


H3R42
H3T45
H3Y41
H2BK34
90°
H3Y41
H3R42
H3T45
H2BK34
H2A
H2B
H3
H4
PDB:1AOI

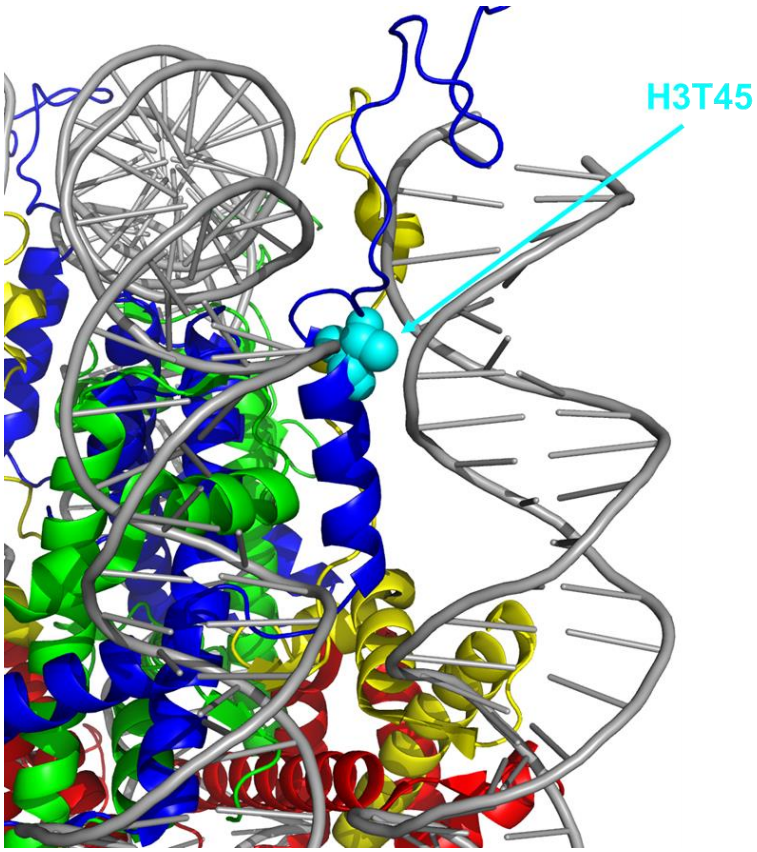
B



C



D



E

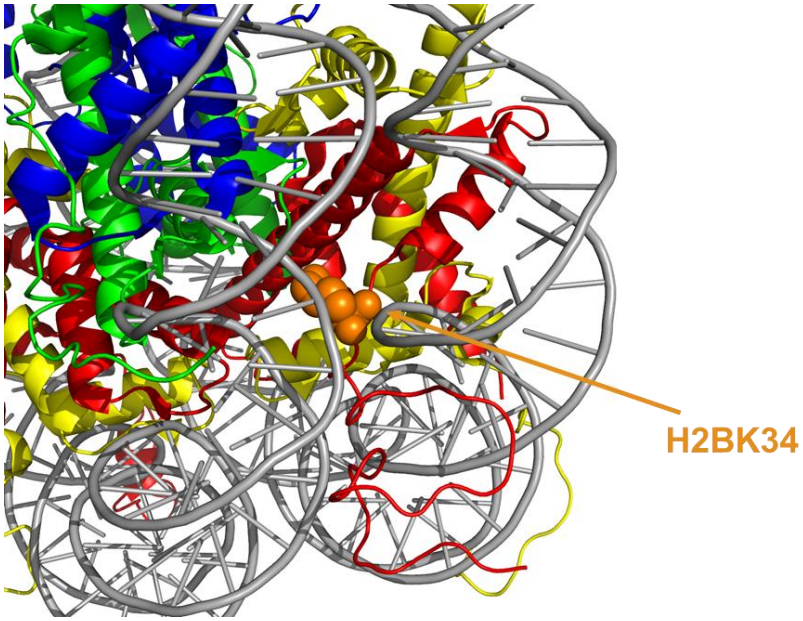


Figure 3.1. Selected potential modification sites (H3Y41, H3R42, H3T45 or H2BK34) are involved in DNA-histone interaction. (A) Overview of the position of selected potential modification sites. Zoom-in view of H3Y41 (B), H3R42 (C), H3T45 (D) or H2BK34 (E) in the context of the nucleosome is indicated by an arrow.

DNA interaction impedes passage of RNA pol II through nucleosomes, histone modifications disrupting this interaction may facilitate gene transcription. This encourages us to investigate if this modification affects nucleosome structure.

Mutagenesis was used to synthesize mimic modification or break the histone-DNA interaction. For example, threonine or tyrosine was mutated to glutamic acid for mimicking phosphorylation (H3Y41E and H3T45E) (Brehove et al. 2015). Arginine was mutated to alanine to block hydrogen bond potential (H3R42A) (Hyland et al. 2011). We reveal that these histone mutants do not affect histone octamer formation. Also, they have no effects on nucleosome reconstitution. SAXS was applied to check if these histone mutants cause a change in shape of mono-nucleosome. According to SAXS data, we claim that H3R42A, H3T45E and H3Y41E alter mononucleosome shape by affecting histone-DNA interaction, shown by an increased D_{max} (maximum interatomic distance) and Rg (Radius of gyration) of nucleosome with these histone mutants. Additionally, we observed that ionic strength also has an effect on nucleosome structure. At 50 mM KCl, nucleosome with the increased D_{max} and Rg indicates that nucleosomal DNA may unwrap from histones. This interpretation was confirmed by enzyme digestion assay. We found that H3T45E significantly facilitates DNA unwrapping from histone octamer, while H3Y41E slightly does. This result was further supported by Micrococcal nuclease (MNase) digestion assay. Our data suggest a potential mechanism for how H3Y41phos prevents heterochromatin formation and how H3T45phos plays a role in DNA replication. These conclusions are consistent with previous results (Simon et al. 2011). Since H2BK34 emerges from two gyres of the nucleosomal DNA superhelix, the introduction of mono-ubiquitin (~8.5 KDa)

at this position may significantly disrupt DNA-histone interaction. However, our MNase digestion data suggest H2BK34ub does not enhance nucleosomal DNA breathing.

3.3 Material and Methods

3.3.1 DNA and histone octamer preparation

147-bp '601' DNA and 207-bp '601' DNA containing nucleosome positioning sequence were prepared as previously described (Dyer et al. 2004). Similarly, histone octamers with WT histone, histone mutants or histone modification were refolded as published before (Dyer et al. 2004).

3.3.2 Nucleosome reconstitution

Nucleosomes were reconstituted by salt dialysis as previously described (Dyer et al. 2004). Nucleosomes used for SAXS and restriction enzyme digestion assay were reconstituted with 147-bp '601' DNA and recombinant *Xenopus laevis* histones, named 147 NCP. Nucleosomes used in MNase digestion assay were reconstituted with 207-bp '601' DNA and recombinant *Xenopus laevis* histones.

3.3.3 Size Exclusion Chromatography with Multi-Angle Light Scattering (SEC-MALS)

Superdex 200 HR 10/30 column (24 ml total volume, GE Healthcare) was run in-line with the MALS instrument (Wyatt technologies). The flow rate was 0.3 ml/min. 100 µl of nucleosomes at 0.3 mg/ml was injected in a buffer containing 20 mM Tris pH 7.5, 1 mM EDTA pH 8.0, 1 mM TCEP. The same samples were used in SAXS at 0 mM or 50 mM KCl. The molecular weight for each sample was calculated using the ASTRA software (Wyatt technologies).

3.3.4 Small Angle X-ray Scattering

All SAXS data was collected at the SIBYLS beam line (12.3.1) at the Advanced Light Source (ALS, Berkeley). Nucleosomes were either stocked in reference buffer (20 mM Tris pH 7.5, 1 mM EDTA, 1 mM DTT) or reference buffer with 50mM KCl to investigate the effect of ionic strength on nucleosome structure. In order to optimize the data quality and minimize radiation damage, exposure series of 0.5, 1, 2, 6 s were performed. Data were processed by PRIMUS (Konarev et al. 2003). The radius of gyration (Rg) was calculated based on low angle data from a Guinier plot (Guinier et al., 1955). The dimension of nucleosomes was estimated by GNOM (Svergun et al. 1987). Ten random molecular envelopes were constructed for each nucleosome by DAMMIN (Svergun and Koch 2003). They were superimposed by DAMSUP (Kozin and Svergun 2001). The average of these 10 random models was calculated by DAMAVER (Konarev et al. 2003). The averaged model was filtered by damfilt. A convex shell of all models were built and visualized as before (Yang et al. 2011).

3.3.5 Restriction enzyme digestion assay

Nucleosomes with canonical histone or histone mutants were assembled with 207-bp '601' DNA. HpaII was used in this assay. The position of the HpaII is shown in Figure 3.7A. 207-bp '601' DNA was used as a positive control. Nucleosomes were stocked in TSC buffer (20 mM Tris pH7.5, 1 mM EDTA, 1 mM DTT, 50 mM KCl). Nucleosomes and 207-bp '601' DNA concentration were kept constant at 50 nM. The reaction was performed in 20 mM Tris pH 7.5, 1 mM EDTA, 1 mM DTT, 50 mM KCl and 1X NEBuffer 1. Reaction time points selected were 0, 5, 10, 20, 30, 60 mins. Reactions were quenched

by termination buffer (40 mM EDTA, 0.2% SDS, 1 mg/ml proteinase K) at room temperature for 30 mins. The quality of reaction was analyzed on a 4% agarose gel. Agarose gels were stained by SYBR gold, and scanned by typhoon.

3.3.6 Micrococcal nuclease digestion assay

Nucleosomes reconstituted with 207-bp DNA that contained a centrally located 147-bp '601' nucleosome positioning sequence were subjected to MNase digestion studies. MNase reactions were performed by combining 30 µl nucleosome (20 ng/µl) or DNA (20 ng/µl) into 2.5 µl BSA (10 mg/ml), 25 µl 10X MNase buffer (NEB), 2 µl (200 U/µl) MNase, and ddH_2O to bring up the total reaction volume to 250 µl. 60 µl reaction mixture was collected at different reaction time points. The reaction was quenched by 5 µl 0.5 M EDTA pH 8.0 and stored on ice. 4.2 µl 10% SDS and 1 µl proteinase K (20 mg/ml) were added into each reaction mixture, and incubated at 55˚C for 30min. DNA was isolated by Phenol-Chloroform extraction. The DNA quantity and length following a MNase digestion of nucleosomes with canonical H3, H3Y41E or H3T45E was analyzed on a 6% native PAGE. The relative mobility (Rf) of each DNA ladder band and MNase digestion product was measured with ImageQuantTL. The Rf and length of each DNA ladder band was correlated and used to calculate the length of each band observed in the MNase reactions.

3.4 Results

3.4.1 Selected histone mutants have no effects on histone octamer stability.

Histone PTMs located at each tail have been substantially investigated. Most of these histone PTMs regulate nucleosome structure through recruitment of chromatin remodelers or histone chaperones (Carvalho et al. 2013, Fischle et al. 2003, Larschan et

al. 2007). In this study, two potential phosphorylation sites (H3Y41 and H3T45) and one potential methylation site (H3R42) were selected. The role of these selected histone PTM mimics in nucleosome structure is investigated.

First, we asked if selected histone mutants affect histone octamer formation. To address this question, histone octamers with selected histone mutants were refolded as described above, and purified by size exclusion column. By doing High Performance Liquid Chromatography (HPLC), we were able to distinguish among histone octamer, H2A-H2B dimer, $(H3\text{-}H4)_2$ tetramer and protein aggregation. In this way, we could determine if histone octamers are refolded. The chromatographic results show that none of the selected histone mutants has an impact on octamer refolding. As expected, histone octamer with variant mutants was eluted at the same volume as WT histone octamer. Then, the quality and composition of histone octamers were analyzed on a 15% SDS gel, and visualized by coomassie staining. Molecular weight of H2B is similar to H2A (~12 KDa), results in their same mobility on a 15% SDS gel. Thus, only three bands attribution to H3, H2A/H2B and H4 were observed on the gel (Figure 3.2). H3Y41E, H3R42A or H3T45E does not significantly alter the mobility of H3 on the gel (Figure 3.2A, B and C). In sum, selected histone mutants do not affect histone octamer formation.

3.4.2 Nucleosome reconstitution with histone mutants

Subsequently, we asked if selected histone mutants may have an impact on nucleosome stability. Phosphorylation at H3Y41 or H3T45 introduces a negative charge to the side chain of each residue. It is possible that H3Y41E or H3T45E disrupts histone-DNA interaction, and thus destabilizing nucleosome. In contrast, di-methylation at H3R42

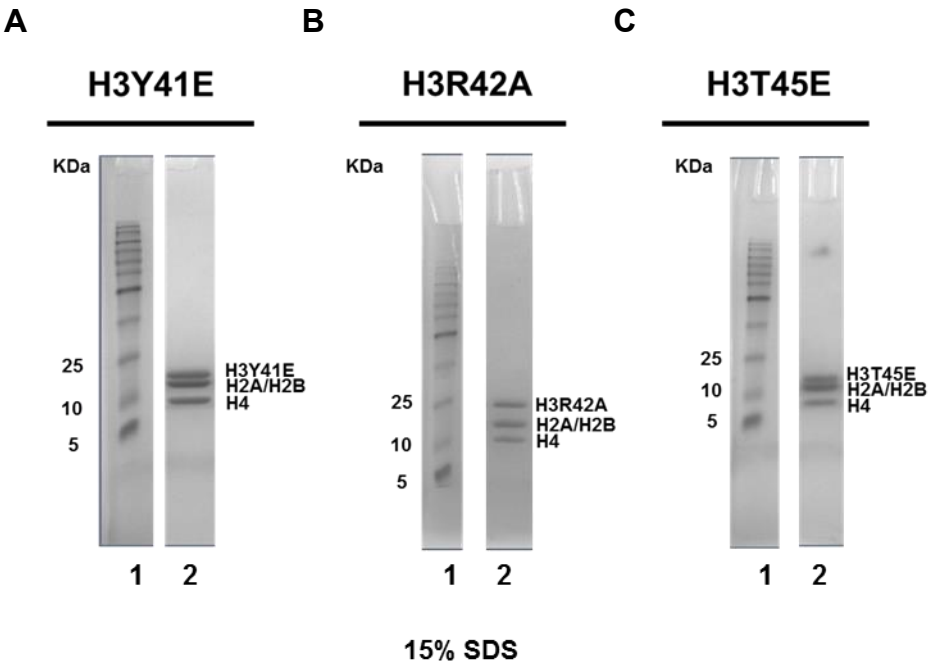


Figure 3.2. H3Y41E, H3R42A or H3T45E does not affect histone octamer integrity. Histone octamers refolded with H3Y41E (A), H3R42A (B) or H3T45E (C) were analyzed on a 15% SDS gel, and visualized by coomassie staining. We found selected histone mutants do not affect the integrity of histone octamer.

preserves the positive charge at its side chain. However, the introduction of methyl group at its side chain may sterically disrupt DNA-histone interaction. Accordingly, we proposed that these selected histone PTM mimics facilitate DNA unwrapping from histone.

To determine if these selected histone mutants affect nucleosome integrity, nucleosomes with canonical octamer or octamer with different histone mutants were reconstituted by salt dialysis. We used a 147-bp '601' nucleosome positioning DNA sequence. Nucleosomes were analyzed on a 5% PAGE, and visualized by EtBr staining (Figure 3.3). Our data suggest that all selected histone mutants could form nucleosomes.

To further confirm that nucleosomes with histone mutants have a full stoichiometry of histones, Size Exclusion Chromatography with Multi-Angle Light Scattering (SEC-MALS) was applied. Nucleosomes were reconstituted with 147-bp '601' DNA and WT or mutant histones. 100 µl of 0.3 mg/ml nucleosome was loaded into SEC-MALS. The light scattering profiles for nucleosomes containing either WT histone or histone mutants were overlaid (Figure 3.4). Two peaks were observed in the light scattering profiles for all nucleosomes. One peak is attributed to nucleosome. The other one is observed in all the samples, even in the buffer control. It may be due to some contamination in the buffer. The calculated MW for different nucleosomes is indicated by arrows. The calculated MW for WT nucleosome with 147-bp DNA is 201 KDa. In contrast, the observed MW of nucleosomes with histone mutants is very closed to 201 KDa. Additionally, the flat dotted line over each nucleosome peak is a strong indication for the monodisperse of nucleosomes. In sum, our results suggest that selected histone mutants do not affect nucleosome integrity.

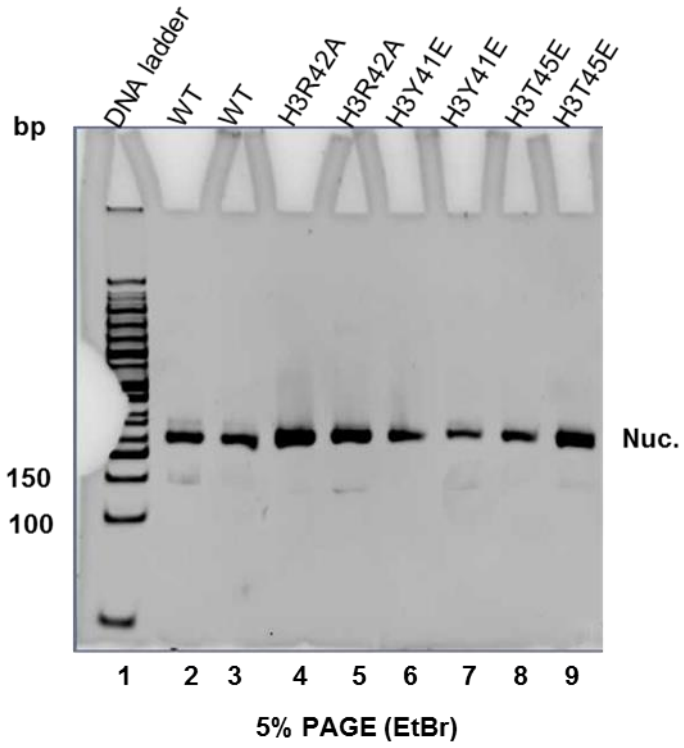


Figure 3.3. H3Y41E, H3R42A or H3T45E does not affect nucleosome reconstitution. Nucleosome with H3Y41E, H3R42A or H3T45E was reconstituted by salt dialysis. The quality of nucleosome was analyzed on a 5% PAGE, and visualized by EtBr staining. We found selected histone mutants do not affect nucleosome reconstitution.

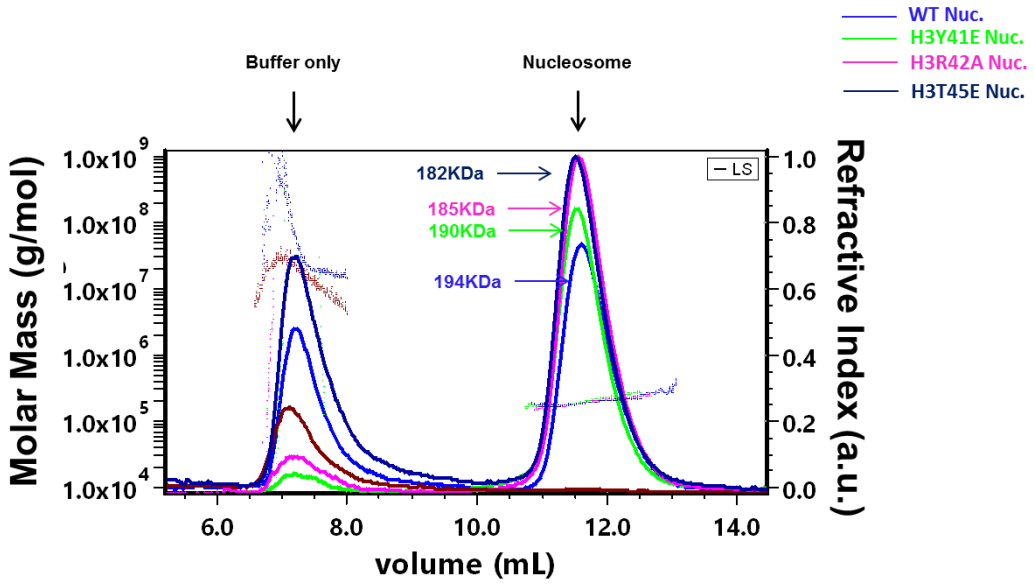


Figure 3.4. The integrity of nucleosome with WT histone and histone mutants was analyzed by SEC-MALS. SEC-MALS profiles for nucleosomes were overlaid. Left y-axis is calculated molar mass, while right y-axis is refractive index. Two peaks were observed in the light scattering profiles for all nucleosomes. One peak is attributed to nucleosome, the other one is attributed to some contamination in the buffer. The calculated MW for different nucleosomes was indicated. The flat dotted line indicates the monodisperse of nucleosomes. This figure has been published.

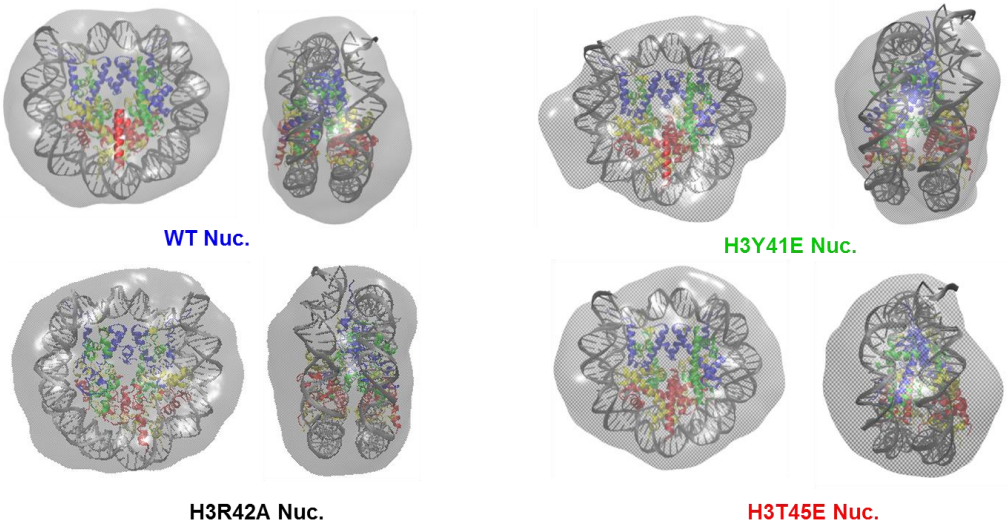
3.4.3 H3Y41E, H3T45E and H3R42A affect the shape of mono-nucleosome.

We determined if the shape of nucleosome is affected by selected histone mutants. Small Angle X-ray scattering (SAXS) was used to check the shape of mononucleosome. With a calculated D_{max} and Rg for nucleosomes, the information about nucleosome shape could be obtained as published before (Yang et al. 2011). To determine the size of molecule, low angle data from a Guinier plot was used to determine the radius of gyration (Rg) (Guinier et al., 1955). The dimension of nucleosomes was estimated by GNOM (Svergun et al., 1992). In order to visualize the conformational change of each nucleosome, ten molecular envelopes were constructed for each nucleosome by DAMMIN (Svergun et al., 1999). They were superimposed by DAMSUP (Kozin et al., 2001). The average of these 10 random models was calculated by DAMAVER (Volkov et al., 2003). The averaged model was filtered by damfilt. A convex shell of all models were built and visualized as before.

In order to investigate if ionic strength has an effect on nucleosome stability, SAXS experiment was performed at either 0 or 50 mM KCl. Increased ionic strength changes the shape of molecular envelop for all selected nucleosomes (Figure 3.5). Also, calculated D_{max} and Rg for nucleosome at 50 mM KCl is much larger than nucleosomes at 0 mM KCl. For example, D_{max} of WT nucleosome at 0 mM KCl is 118 Å, while D_{max} is increased to 135 Å at 50 mM KCl (Table 3.1 and 3.2). It indicates that increased ionic strength may facilitate DNA unwrapping from histones.

Next, we asked if histone mutants have an effect on nucleosome stability. The overall scattering curve is very similar between WT nucleosome and nucleosome with histone

A



B

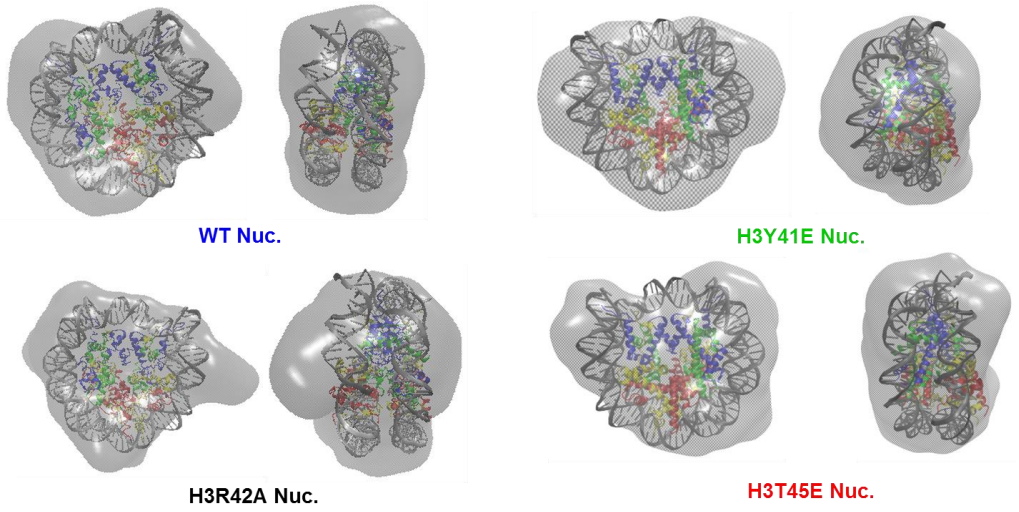


Figure 3.5. The molecular envelop for nucleosomes contains either WT H3 or H3 mutants. Ab initio was calculated for nucleosomes at 0 mM KCl (A) or 50 mM (B). The shell was superimposed onto the crystal structure of the nucleosome (PDB:1AOI). Increased ionic strength (0 mM to 50 mM KCl) caused significant shape change of molecular envelop. Under same ionic strength, overall shape of the molecular envelop for different nucleosomes is very similar. Slight difference was observed between WT nucleosome and nucleosome with histone mutants. This figure has been published.

Table 3.1. SAXS analysis of nucleosomes with WT H3, H3Y41E, H3R42A or H3T45E at 0 mM KCl

Sample name	Radius of gyration (Rg) (Å)	Dmax (Å)
WT nuc.	42.2	118
H3Y41E nuc.	43.3	125
H3R42A nuc.	42.1	118
H3T45E nuc.	42.4	119

Table 3.2. SAXS analysis of nucleosomes with WT H3, H3Y41E, H3R42A or H3T45E at 50 mM KCl

Sample name	Radius of gyration (Rg) (Å)	Dmax (Å)
WT nuc.	44.2	135
H3Y41E nuc.	44.9	140
H3R42A nuc.	44.2	160
H3T45E nuc.	44.5	160

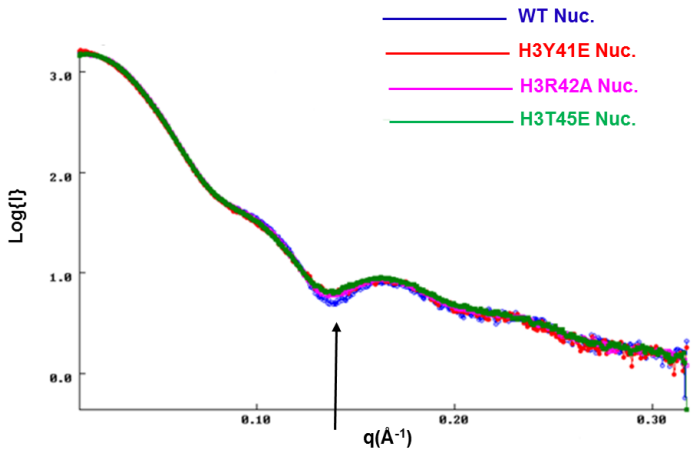
mutants. However, the region indicated by arrow has slight difference between WT nucleosomes and nucleosome with mutants at both salt concentrations (0 mM and 50 mM KCl) (Figure 3.6 A and B). It suggests that selected histone mutants alter the shape of nucleosome (Figure 3.5). At 0 mM KCl, WT nucleosome and nucleosome with histone mutants have similar calculated D_{max}. In contrast, nucleosomes with H3R42A and H3T45E have much larger D_{max}, while nucleosomes with H3Y41E has slightly larger D_{max} than D_{max} of WT nucleosomes at 50 mM KCl (Table 3.2). For example, at 50 mM KCl, calculated D_{max} of WT nucleosome is 135 Å, while calculated D_{max} for nucleosomes with H3R42A or H3T45E is 160 Å. Together, we revealed that both ionic strength and selected histone mutants may affect nucleosomal DNA interaction with histones.

3.4.4 Histone mutants (H3Y41E and H3T45E) enhance DNA accessibility.

Our SAXS data suggest that histone mutants affect nucleosome stability. To further confirm this result, restriction enzyme digestion assay was applied. This assay was used to investigate if histone mutants facilitate nucleosomal DNA breathing (Moyle-Heyrman et al., 2011).

Nucleosomes with canonical histone or histone mutants were assembled with 207-bp '601' DNA. HpaII was utilized in this assay. The recognition site of HpaII was 34-bp from one end of 207-bp DNA, which is protected by H3 αN helix (Figure 3.7A). This recognition site is not accessible in the context of nucleosome. Assuming histone mutants increase nucleosomal DNA breathing, then HpaII could rapidly cleave nucleosomal DNA at its recognition, results in two DNA fragments (34 and 173-bp). The reaction mixtures were incubated at 30 ˚C. The reaction was quenched at different time points (0, 5, 10, 20, 30

A



B

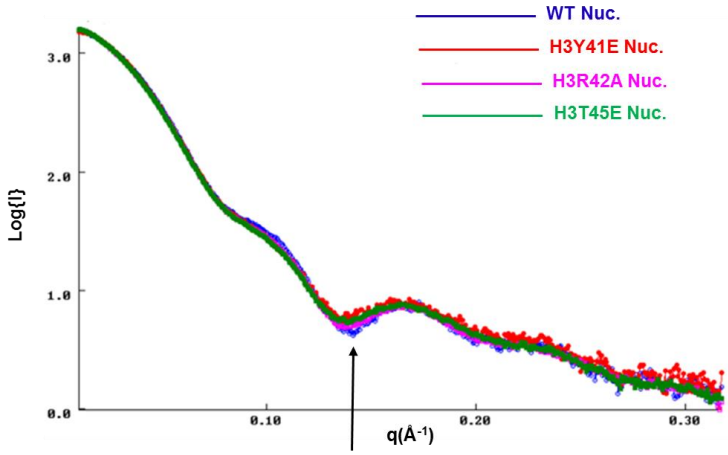
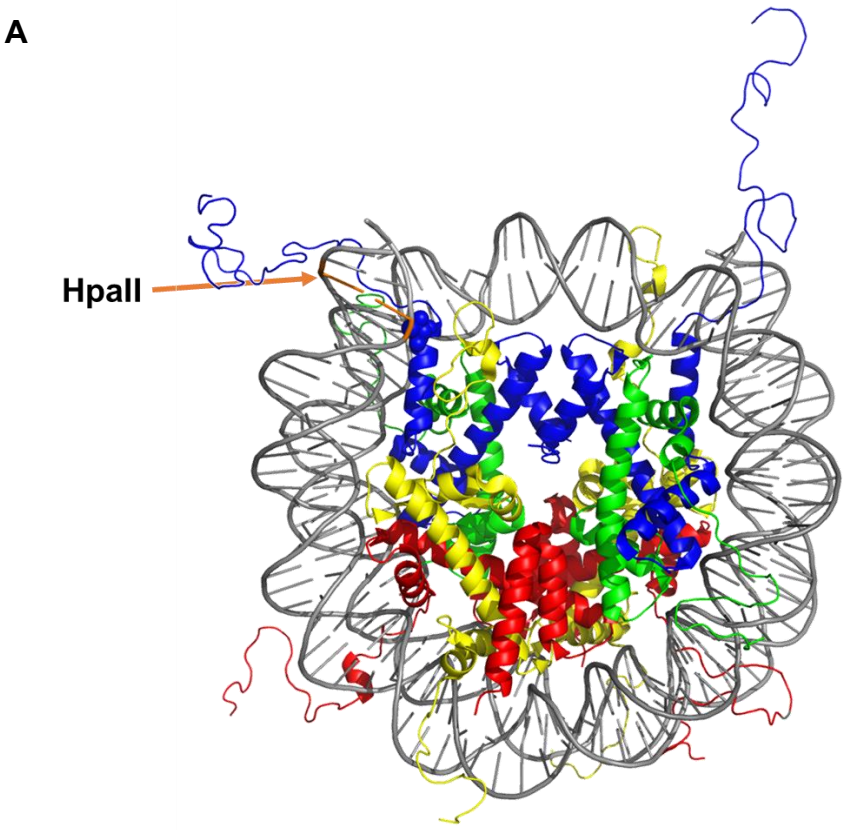
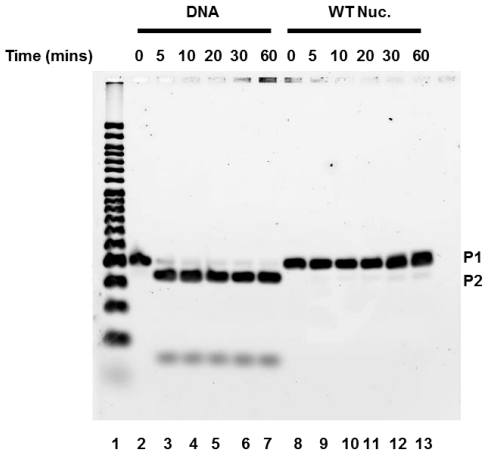


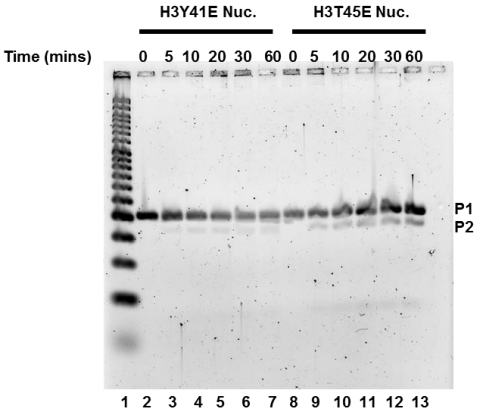
Figure 3.5. Overview of scattering curve for nucleosome with WT histone or histone mutants. The scattering profiles for nucleosomes with WT or histone mutants at 0 mM KCl (A) or 50 mM KCl (B) were overlapped. Notably, the shape of scattering curve between WT nucleosome and nucleosome with different histone mutants is very similar. Only slight difference was observed. It indicates that histone mutants alter the shape of mononucleosome. This phenomenon is not dependent on ionic strength.


A
HpaII

B


DNA
WT Nuc.
Time (mins)
0 5 10 20 30 60 0 5 10 20 30 60
P1
P2
1 2 3 4 5 6 7 8 9 10 11 12 13

C


H3Y41E Nuc.
H3T45E Nuc.
Time (mins)
0 5 10 20 30 60 0 5 10 20 30 60
P1
P2
1 2 3 4 5 6 7 8 9 10 11 12 13

D

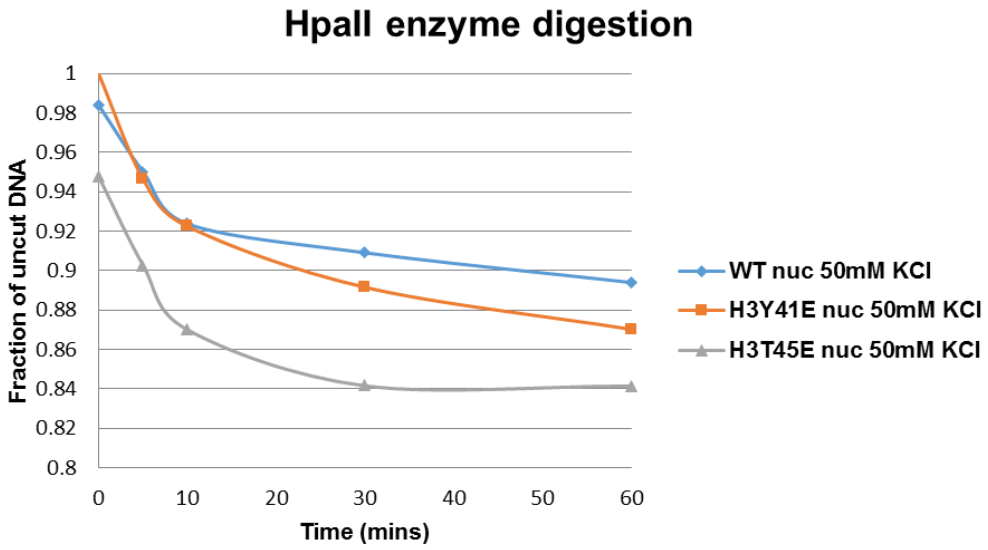


Figure 3.7. H3T45E facilitates 'DNA breathing', while H3Y41E slightly affects nucleosomes stability. (A) The restriction enzyme (HpaII) recognition site is indicated in the context of nucleosome. (B) and (C) The quality of HapII digestion for nucleosomes with canonical H3, H3Y41E or H3T45E was analyzed on a 4% agarose gel. The gel was visualized by SYBR Gold staining and scanned by typhoon. The reaction was performed at 50 mM salt. Time points were 0, 5, 10, 20, 30, 60 mins. Nucleosome concentration was kept at 50 nM, and mixed with 1000 U/ml HapII. (D) Quantitative analysis of the digestion reaction resolved in the gel (B) and (C). Fraction of protected full length DNA= P1/(P1+P2). The intensity of each band was measured with 1D gel analysis function of ImageQuantTL software. The same reaction was done three times. The same trend was maintained.

and 60 mins). The digested DNA fragment was analyzed on a 4% agarose gel (Figure 3.7B-C). The gel was visualized by SYBR Gold staining. The gel was quantified by ImageQuantTL. The fraction of full length DNA was plotted as a function of digestion time (Figure 3.7D). Free DNA was rapidly digested in the presence of HpaII. Two DNA fragments (34 and 173-bp) were observed as expected. In contrast, nucleosome with canonical histones was resistant to HpaII, due to DNA-histone interaction. After 60 mins digestion reaction, only a small amount of nucleosomal DNA was digested. Compared to WT nucleosome, nucleosome with H3Y41E is slightly more sensitive to HpaII. In contrast, nucleosome with H3T45E was the most sensitive to HpaII. After 10 mins reaction time, ~20% H3T45E nucleosomal DNA was digested. Thus, both H3Y41E and H3T45E facilitate nucleosomal DNA breathing. The experiment was done in three biological replicates. The trend was conserved.

3.4.5 Micrococcal nuclease digestion assay for WT, H3Y41E and H3T45E nucleosome.

Our restriction enzyme digestion data suggest that both H3Y41E and H3T45E facilitate nucleosomal DNA unwrapping from histones. To further confirm that selected histone mutants promote DNA unwrapping from histones, MNase digestion assay was performed. MNase cleaves linker DNA, but pauses due to histone-DNA interaction, and thus protects ~150-bp DNA fragment. Assuming that selected histone mutants cause transient unwrapping of DNA from the histone octamer surface, less nucleosomal DNA will be protected. 207 nucleosome was subjected to MNase digestion. 30 µl nucleosome containing either WT and histone mutants (20 ng/µl) or 207-bp free DNA (20 ng/µl) was mixed with MNase (400 U). The reaction mixtures were incubated at 37 °C for 10 mins.

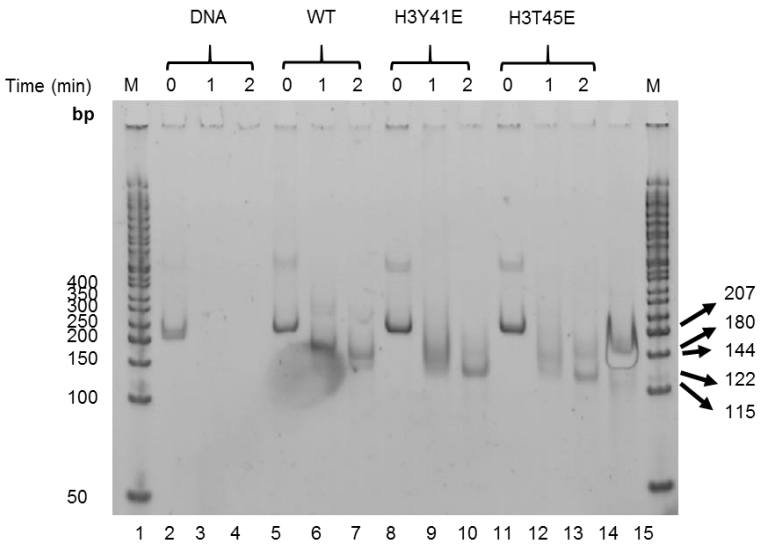
The reaction was quenched by EDTA. The protected DNA fragments were analyzed on a 6% PAGE (Figure 3.8A). The gel was visualized by SYBR Gold staining and scanned by typhoon. The gel was quantified by ImageQuantTL. The length of protected DNA was plotted as a function of digestion reaction time (Figure 3.8B). In WT nucleosomes, a single 165-bp band was observed after 60 seconds of digestion reaction. ~140-bp DNA fragment was observed after 120 seconds of digestion reaction. It is due to the nucleosome boundaries. In contrast, nucleosome containing either H3Y41E or H3T45E does not cause MNase pausing sites, and thus no ~150-bp DNA fragment was observed. Instead, a shorter DNA fragment was observed after 60 seconds digestion reaction. ~120-bp DNA fragment was only observed for nucleosome with either H3Y41E or H3T45E after 120 seconds digestion reaction (Fig 3.8A-B). Again, it indicates that H3Y41E and H3T45E facilitate DNA unwrapping from the surface of histone octamer.

Together, MNase digestion data are consistent with the results obtained from SAXS and restriction enzyme digestion assay. All the evidence supports our hypothesis that histone modifications from entry-exit region of nucleosome have effects on nucleosomal DNA breathing.

3.4.6 H2BK34ub does not affect either histone octamer refolding or nucleosome reconstitution.

We show that selected histone modification mimics from entry-exit nucleosome region facilitates nucleosomal DNA breathing. Next, we asked if histone modifications from H2B N-terminal tail have similar effects on nucleosome stability. H2BK34ub was selected to test this hypothesis.

A


DNA
WT
H3Y41E
H3T45E
Time (min)
M 0 1 2 0 1 2 0 1 2 0 1 2 M
bp
400
350
300
250
200
150
100
50
207
180
144
122
115
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

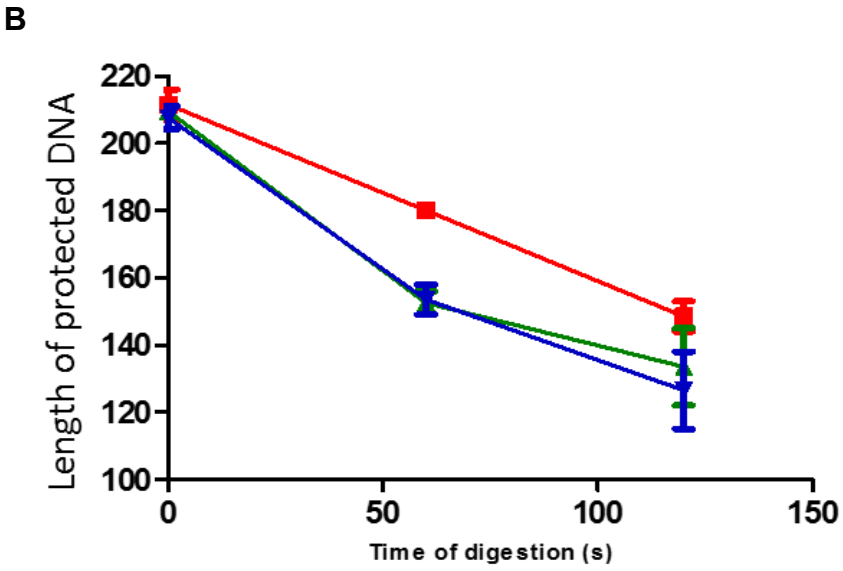


Figure 3.8. Micrococcal nuclease (MNase) digestion of WT, H3Y41E or H3T45E nucleosomes. (A). Nucleosomes with 207-bp '601' DNA and histone octamer containing either WT H3, H3Y41E or H3T45E were digested with MNase. The digestions were quenched at 0, 60 and 120 s. Proteinase K was added into the reaction mixture. The length of protected DNA fragment was then analyzed on a 6 % polyacrylamide gel. The gel was visualized by SYBR Gold staining and scanned by typhoon. (B). The length of protected DNA fragment is presented as a function of digestion time. The migration distance of each band (A) was measured with 1D gel analysis function of ImageQuantTL software. This figure has been published.

Firstly, we attempted to test if H2BK34ub affects histone octamer refolding and nucleosome reconstitution. Histone octamer was refolded as mentioned above. The quality and composition of histone octamers were analyzed on a 15% SDS gel, and visualized by coomassie staining (Figure 3.9). Four bands attribution to H2A, H2BK34ub, H3 and H4 were observed on the gel, because mono-ubiquitination at H2BK34 significantly changes the mobility of H2B. The molecular weight of H2BK34ub is about 22 KDa. Next, we asked if H2BK34ub affects nucleosome reconstitution. Nucleosomes with H2BK34ub were reconstituted as mentioned above. Nucleosomes were analyzed on a 5% PAGE, and visualized by EtBr staining (Figure 3.10). H2BK34ub significantly alters the mobility of mononucleosome, likely due to the mono-ubiquitin molecule. In sum, H2BK34ub does not affect either octamer formation or nucleosome reconstitution.

3.4.7 Micrococcal digestion assay for H2BK34ub nucleosome

Having shown that H2BK34ub does not affect the integrity of histone octamer and nucleosome reconstitution, subsequently, we investigated if H2BK34ub facilitates transient nucleosomal DNA unwrapping. MNase digestion assay was performed as above. Nucleosomes with H2BK34ub and 147-bp '601' DNA were subjected to MNase digestion assay. The protected DNA fragments were analyzed on an 8% PAGE, and visualized by EtBr. staining (Figure 3.11). Similarly, we observed that free DNA was totally digested after 30 seconds digestion reaction. In contrast, DNA in WT nucleosome is slightly digested after 120 seconds digestion reaction. Compared to WT nucleosome, nucleosome with H2BK34ub has similar MNase digestion pattern. It indicates that H2BK34ub nucleosome behaves similarly as WT nucleosome in the MNase digestion assay. MW of mono-ubiquitin molecule is ~8.5 KDa. H2BK34 is located at H2B N-terminal

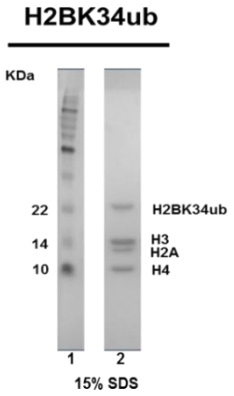


Figure 3.9. H2BK34ub does not affect histone octamer integrity. Histone octamer refolded with H2BK34ub was analyzed on a 15% SDS gel, and visualized by coomassie staining. We found selected histone modification does not affect the integrity of histone octamer.

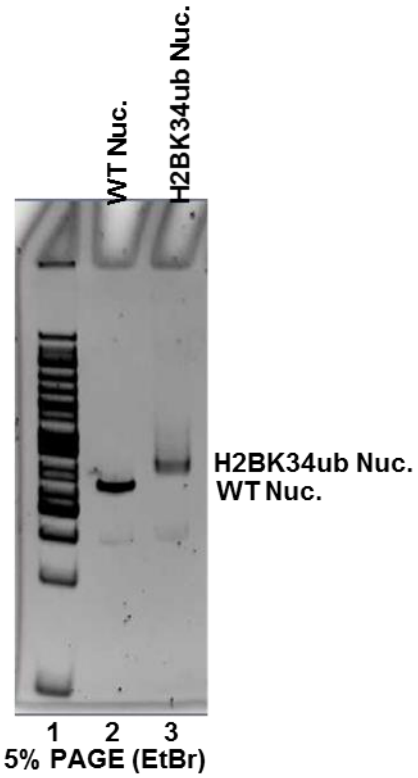


Figure 3.10. H2BK34ub does not affect nucleosome reconstitution. Nucleosome with H2BK34ub was reconstituted by salt dialysis. The quality of nucleosome was analyzed on a 5% PAGE, and visualized by EtBr staining. We found selected H2BK34ub does not affect nucleosome reconstitution. However, we notice that H2BK34ub significantly changes the mobility of mono-nucleosome. It may be due to the introduction of mono-ubiquitin molecule to nucleosome.

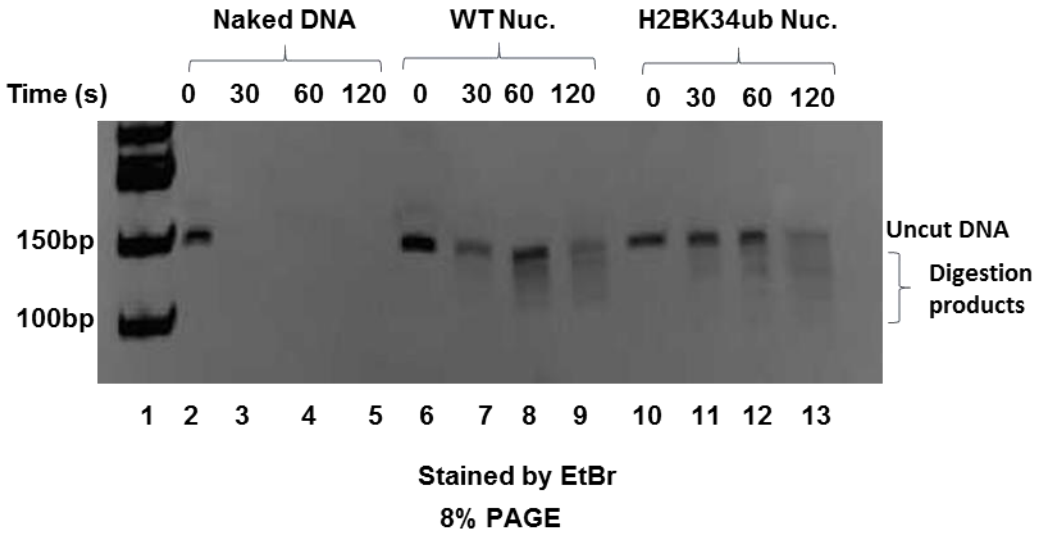


Figure 3.11. Micrococcal nuclease digestion of H2BK34ub nucleosome. Nucleosome was reconstituted with 147-bp and histone octamer with H2BK34ub. MNase digestion assay was performed as above. The length of protected DNA fragment was analyzed on an 8% PAGE. The gel was visualized by SYGR Gold staining. Interestingly, nucleosome with H2BK34ub has similar MNase digestion pattern as WT nucleosome. It indicates that H2BK34 does not affect nucleosomal DNA breathing.

tail between two nucleosomal DNA strands. Surprisingly, the introduction of mono-ubiquitin at H2BK34 does not affect DNA breathing.

3.5 Discussion

Histone PTMs play an important role in nucleosome structure (Lu et al. 2008, Luebben et al. 2010, Potoyan and Papoian 2012). Decades ago, Bode and his colleagues suggested that hyperacetylated histones increased nucleosomal DNA accessibility, which facilitated protein binding to target DNA (Bode et al. 1983). This is the first evidence to support histone modifications disrupt DNA-histone interaction. Similarly, nucleosomes with hyperacetylated histones has different the hydroxyl radical footprinting, compared with nucleosomes with unmodified histones (Bauer et al. 1994). It indicates that DNA-histone interaction patterns were changed due to the hyperacetylated histones. All these evidence suggest that histone modifications have effects on histone-DNA interaction in nucleosomes. In this chapter, we selected several potential histone modification sites (H3Y41, H3R42 and H3T45) from nucleosomal entry-exit region and one site from H2B N-terminal tail (H2BK34). The effects of three histone modification mimics and one histone modification on nucleosome integrity and stability have been investigated. Our biochemical studies suggest that selected histone mutants or histone modification does not affect the integrity of the histone octamer and nucleosome. Since these histone mutants are located at the surface of histone octamer, we do not expect they have effects on histone octamer or nucleosome assembly.

This result was subsequently confirmed by SEC-MALS. Based on the light scattering profile, we found the observed MW of nucleosomes with histone mutants is similar to the

calculated MW of WT nucleosomes, a strong indication that selected histone mutants do not affect the integrity of nucleosome.

To explore this idea that selected histone mutants affect histone-DNA interactions, SAXS was applied. According to SAXS data, ionic strength affects the mononucleosome structure. For example, at 50 mM KCl, D_{max} of WT nucleosomes is much larger than D_{max} of WT nucleosome at 0 mM KCl. Additionally, we found that D_{max} of nucleosomes with H3R42A or H3T45E (mimic phosphorylation) is much larger than that of WT nucleosome at 50 mM KCl. Intuitively, we proposed that selected histone mutants affect mononucleosome stability via facilitating DNA unwrapping from histone octamer.

Our hypothesis was supported by restriction enzyme (HpaII) digestion assay. At 50 mM KCl, the protected DNA fractions for H3Y41E nucleosomes is slightly less than that for WT nucleosomes, and more than that for H3T45E nucleosomes. Less nucleosomal DNA is protected due to highly frequent and short-lived conformation change of nucleosomes. This result was consistent with our SAXS data.

To further confirm that selected histone mutants have effects on nucleosomal ‘DNA breathing’, MNase digestion assay was applied. We observed that ~150-bp protected DNA fragment was observed for WT nucleosome after 120 seconds digestion reaction, due to histone-DNA interaction at the edge of mononucleosome. In contrast, shorter nucleosomal DNA (~120-bp) in either H3Y41E or H3T45 nucleosome was protected after 120 seconds digestion reaction. It indicates that selected histone mutants facilitate transient DNA unwrapping from the surface of histone octamer. The same MNase digestion assay was performed to nucleosome with H2BK34ub and 147-bp DNA.

Surprisingly, we found that H2BK34ub does not affect nucleosome stability, as defined by this assay. Nucleosome with H2BK34ub has similar MNase digestion pattern as WT nucleosome.

In sum, our biochemical data suggest that selected histone modification mimics affect nucleosome stability via facilitating DNA breathing. Histone modifications regulate nucleosome structure in a network (Brehove et al. 2015). Our future plan is to combine different histone modifications to investigate if different combination patterns have distinct effects on nucleosome structure.

3.6 Acknowledgement

We thank Dr. Michael G. Poirier for generously providing us with H3T45E and H3Y41E histone. We also thank Dr. Hataichanok Scherman for providing histones. We are grateful for Dr. Yali Dou providing H2BK34ub histones. Dr. Mark van der Woerd helped us to collect and analyze SAXS data and SEC-MAL data. We thank Dr. Serge Bergeron and Pam Dyer for discussion.

CHAPTER 4

SUMMARY AND FUTURE DIRECTIONS

My dissertation focuses on how histone chaperone (FACT) regulates nucleosome structure. FACT was discovered as a transcription factor that facilitates gene transcription (Belotserkovskaya and Reinberg 2004). Here, we revealed that hFACT binds hH2A-H2B and hH3-H4 with similar affinity. It indicates that FACT is not only a H2A-H2B chaperone, but also a H3-H4 chaperone.

FACT is highly conserved in eukaryotes (Orphanides et al. 1999). yFACT binds two H2A-H2B dimers via two acidic domains in ySpt16 and Pob3, while hFACT only binds one H2A-H2B dimer via acidic domain in hSpt16 (Kemble et al. 2015, Winkler et al. 2011). Two acidic domains in ySpt16 and Pob3 are conserved in hFACT, which encourages us to re-evaluate the stoichiometry of hFACT with H2A-H2B dimer. Our AUC data suggest that hFACT may only bind one H2A-H2B dimer. The reason for this discrepancy is not clear. However, it has been shown that Pob3 F512 is indispensable for Pob3 interaction with H2A-H2B (Kemble et al. 2015). Single-point mutation of this residue totally abolishes Pob3-H2A-H2B interaction. This residue is not conserved in hSSRP1, although the acidic domain is conversed, which may result in hFACT binding one H2A-H2B dimer. The stoichiometry of hFACT-(H3-H4) was also determined by AUC. However, hFACT-(H3-H4) forms aggregates, which may be due to nonspecific interaction between Spt16-CTD and H3-H4. Additionally, it was suggested that FACT has the potential to bind H2A-H2B and H3-H4 simultaneously (Kemble et al. 2015, Tsunaka et al. 2016). By using AUC-FDS, we show that hFACT binds a histone hexamer composed of one H2A-H2B dimer and one

$(H3\text{-}H4)_2$ tetramer. This result also implies that H2A-H2B dimer facilitates FACT interaction with $(H3\text{-}H4)_2$ tetramer.

FACT-histones interaction has been well characterized. However, the exact role of FACT in nucleosome structure is still unknown. By using an in vitro gel-based nucleosome disassembly assay, we show that FACT per se does not disassemble nucleosomes. Also, FACT does not bind to mononucleosome. This interpretation is confirmed by AUC data. It indicates that FACT is not a nucleosome disassembly factor. This result is also consistent with crystal structure of Spt16-CTD-H2A-H2B and Spt16MD-AID-H3-H4, which suggests that FACT binding site at histones are blocked by either DNA or histones in the context of nucleosome (Kemble et al. 2015, Tsunaka et al. 2016). Similarly, it has been shown that FACT efficiently facilitates RNA pol II overcoming H3-H4-DNA interaction, but not H2A-H2B-DNA interaction (Bondarenko et al. 2006). In agreement, our in vitro transcription data suggest that FACT does not facilitate RNA pol II progression through the pause sites caused by H2A-H2B-DNA interaction and H3-H4-DNA interaction. All these evidence suggest that FACT does not initialize nucleosome disassembly.

Next, we investigated if FACT facilitates nucleosome assembly. By using an in vitro gel-based nucleosome assembly assay, we were able to dissect which step of nucleosome assembly FACT is involved in. We found that FACT is a robust tetrasome assembly factor. Besides, FACT also facilitates H2A-H2B deposition onto tetrasome and hexasome, and thus assembles nucleosomes. This hypothesis is confirmed by MNase digestion assay. However, the mechanism of how FACT facilitates tetrasome assembly and H2A-H2B deposition is still unknown. Since FACT prevents nonspecific H2A-H2B-DNA interaction,

we speculate that FACT facilitates H2A-H2B deposition by preventing nonspecific histone-DNA interactions.

Our data suggest that FACT is a nucleosome assembly factor, which is very consistent with genetic data from several labs. However, this does not explain how FACT facilitates gene transcription. It has been proposed that FACT could increase the global accessibility of nucleosomal DNA by tethering nucleosome components together (Kemble et al. 2015, Kulaeva et al. 2013, Rhoades et al. 2004, Ruone et al. 2003). However, no direct evidence supports this model. Here, we observed that H2A-H2B dimer facilitates FACT interaction with tetrasomal H3-H4, and forms a supershifted complex, which is comprised of a histone hexamer, DNA and FACT. Compared to nucleosome, DNA does not tightly wrap around histones in the supershifted complex. All these results suggest that FACT is able to form an unstable histone-DNA complex by tethering partial components of nucleosomes.

Histone mutant (H4Y98H) disrupting dimer-tetramer interaction does not only affect FACT holding nucleosome components, but also impedes FACT binding a histone hexamer. This result implies that H2A-H2B interacts with H3-H4 as FACT binding a histone hexamer. Since H2A-H2B only does not bind to H3-H4 under physiological salt condition, FACT facilitates H2A-H2B and H3-H4 interaction. Intuitively, we propose that FACT interacts with H2A-H2B first, and subsequently folds over the H2B-H4 interface, and results in interaction with distal H2A docking site at the surface of H3-H3'-H4' and proximal H4 L1 loop. In this way, FACT is able to stabilize dimer-tetramer interactions, and also prevents the second H2A-H2B dimer binding to the hexamer by blocking the H2A docking site. This model suggests that FACT disrupts H3-H4-DNA interactions

around the nucleosome dyad, which is in the line with previous results (Hsieh et al. 2010, Ujvári et al. 2008). This model is also supported by our MNase digestion assay, which implies that DNA in the supershifted complex is not tightly wrapped around histones. Compared to hFACT, yFACT is able to bind to nucleosome via an independent subunit (Nph6), which is a member of HMGB family (Stillman 2010). It has been shown that yFACT binds to nucleosome, and resulting nucleosomes are highly sensitive to endonucleases (Formosa et al. 2001, Ruone et al. 2003, Takahata et al. 2009).

Together, our results provide mechanistic insight into how FACT regulates nucleosome structure. FACT maintains nucleosome structure by facilitating tetrasome assembly and deposition of H2A-H2B onto tetrasomes or hexasomes. Meanwhile, FACT also has potential to tether nucleosome components, and thus forming an unstable complex. In order to hold nucleosome components, FACT needs to bind to free H2A-H2B first, and then interact with tetrasomal H3-H4. This suggests that interaction of H2A-H2B is the 'trigger' for FACT holding nucleosome components. It may explain why interaction of H2A-H2B is crucial for FACT activity (Belotserkovskaya et al. 2003).

However, it is still not clear how histones interact with DNA in this supershifted complex. Point mutation at H3 αN helix disrupting dimer-tetramer interaction impedes FACT tethering nucleosome components. It indicates that H3 αN helix may interact with DNA in the supershifted complex, as it does in the nucleosome (Luger et al. 1997).

In chapter 2, we also investigate how FACT activity is regulated. Previous studies suggest that H2BK120ub facilitates FACT function during transcription (Pavri et al. 2006), and we therefore investigated the contribution of mono-ubiquitination at either H2A or H2B in

FACT-H2A-H2B interaction and FACT assembly activity was determined in vitro. Our data suggest that mono-ubiquitination at H2A or H2B does not affect either FACT-H2A-H2B interaction or FACT assembly activity.

Finally, the role of FACT in gene transcription was investigated. We found that FACT per se slightly stimulates gene transcription. In the presence of FACT, we observed two RNA pol II pausing sites, which are caused by H2A-H2B-DNA interaction (~86-bp) and H3-H4-DNA interaction (~105-bp). This indicates that FACT does not facilitate RNA pol II overcoming these nucleosome barriers. Again, this result implies that FACT per se does not initialize nucleosome disassembly. However, the presence of these pause sites does not decrease the amount of full length transcripts. Instead, full length transcripts are increased moderately along with increased amount of these two pause sites, which suggests that FACT slightly facilitates gene transcription. Also, increased amount at two pausing sites indicates that histones remain at the same position on DNA, which supports the idea that FACT does not completely displace all core histones. How does FACT facilitate gene transcription without displaces all core histones? We propose that FACT may transiently disrupt several histone-DNA interactions. It was suggested that three strong histone-DNA interaction regions impede RNA pol II progression efficiency (Bondarenko et al. 2006). Two of them are due to two H2A-H2B dimers interaction with nucleosomal DNA closed to the edge of nucleosome, one is attributed to H3-H4-DNA interaction around the nucleosomal dyad. Notably, we did not observe any pol II pausing sites around nucleosome dyad or distal H2A-H2B-DNA interaction region in the presence of FACT. Thus, these pausing sites may be removed by FACT. Perhaps, FACT tethers nucleosome components as RNA pol II approaches and reassembles nucleosome after

passage of pol II. In this way, FACT could facilitate RNA pol II activity without the depletion of histones. Again, mono-ubiquitination at H2A or H2B does not affect FACT function in gene transcription either.

Future effort should be put into understanding the mechanism how FACT assembles nucleosomes. The contribution of each domain of FACT in its assembly activity should be investigated. Additionally, it is still not clear how FACT is recruited to gene coding regions. Possibly, histone modifications are involved in the recruitment of FACT by facilitating FACT-histone interaction. Accordingly, we intend to investigate if histone modifications affect FACT-histone interaction. Similarly, the role of different histone variants in FACT activity should also be investigated. To follow up on the idea that the H3 αN helix may interact with DNA in the FACT assembled supershifted complex, point mutations disrupting histone-DNA interactions at this region will be selected to test this hypothesis. We also intend to obtain high resolution structural information of the FACT assembled supershifted complex. Our plan is to assemble the supershifted complex by using tetrasome with 79-bp DNA. The supershifted complex will be purified via affinity chromatography, and then subjected to cryo-electron microscopy or crystallization. We will test whether FACT-assembled supershifted complex is able to facilitate gene transcription.

The second part of my dissertation focused on how histone PTMs affect nucleosome structure. We selected several potential histone modification sites. Most of them (H3Y41, H3R42 and H3T45) are located at the entry-exit region of nucleosome, while one (H2BK34) is located at H2B N-terminal tail. H3Y41 and H3T45 are two potential phosphorylation sites, while H3R42 is a potential methylation site. Histone modifications

occurring at these sites play an important role in DNA replication and gene transcription (Baker et al. 2010, Dawson et al. 2009, Hyland et al. 2011). Our biochemical data suggest that selected histone PTM mimics affect the shape of mononucleosome. We proposed that these histone PTM mimics facilitate DNA unwrapping from histone octamer. This hypothesis was supported by restriction enzyme and MNase digestion assay. H3T45E significantly facilitates DNA unwrapping from the surface of histone octamer, while H3Y41E slightly facilitates 'DNA breathing'. These results support the hypothesis that histone modifications from the nucleosome entry-exit region affect DNA-histone interaction. Subsequently, we tested if histone modifications from H2B N-terminal tail also have effects on DNA-histone interactions. H2BK34ub was selected to test this hypothesis. Our biochemical data suggest that mono-ubiquitination at H2BK34 does not facilitate DNA unwrapping from histone octamer. In order to obtain more structural information, we attempted to solve the crystal structure of nucleosomes with H2BK34ub. Unfortunately, no crystals were grown under the condition we tested.

Only several forms of histone modifications have been highlighted in this dissertation. Countless other histone modifications at many different residues in the histones have been discovered, such as sumoylation, ADP-ribosylation and glycosylation (Kelly and Hart 1989, Shiio and Eisenman 2003, Ueda et al. 1975). All these histone modifications may have effects on nucleosome stability. For example, ADP-ribosylation introduces two negative charges to the side chain of modified residues, which may disrupt the histone-DNA interactions in a similar way to phosphorylation. Besides, the size of ADP-ribose is proximally equal to 5 amino acids. Thus, ADP-ribosylation of histones may have more dramatic effects on chromatin structure than phosphorylation of histones. Indeed, it has

been shown that chromatin with ADP-ribosylated histones is more sensitive to MNase than unmodified chromatin, which indicates an ‘open’ chromatin structure (Perez-Lamigueiro and Alvarez-Gonzalez 2004). Overall, considerable effort has been invested into investigating the biological role of histone PTMs. However, many questions still need to be answered: why do some histone modifications coexist? Do they affect nucleosome stability cooperatively? How are histone modifications inherited? In order to address these questions, the effects of different histone PTM combinations on nucleosome stability or higher order chromatin structure should be tested. Also, histone PTMs from different regions of nucleosome should be tested to see how histone PTMs regulate nucleosome structure.

REFERENCES

Allan, J., P. G. Hartman, C. Crane-Robinson and F. X. Aviles (1980). The structure of histone H1 and its location in chromatin. Nature. **288:** 675-679.

Andrews, A. J., X. Chen, A. Zevin, L. A. Stargell and K. Luger (2010). "The histone chaperone Nap1 promotes nucleosome assembly by eliminating nonnucleosomal histone DNA Interactions." Mol. Cell **37**: 834-842.

Andrews, A. J., X. Chen, A. Zevin, L. a. Stargell and K. Luger (2010). "The histone chaperone Nap1 promotes nucleosome assembly by eliminating nonnucleosomal histone DNA interactions." Molecular cell **37**: 834-842.

Andrews, A. J., G. Downing, K. Brown, Y. J. Park and K. Luger (2008). "A thermodynamic model for Nap1-histone interactions." J Biol Chem **283**(47): 32412-32418.

Arents, G. and E. N. Moudrianakis (1995). "The histone fold: a ubiquitous architectural motif utilized in DNA compaction and protein dimerization." Proc Natl Acad Sci U S A **92**(24): 11170-11174.

Baker, S. P., J. Phillips, S. Anderson, Q. Qiu, J. Shabanowitz, M. M. Smith, J. R. Yates, D. F. Hunt and P. a. Grant (2010). "Histone H3 Thr 45 phosphorylation is a replication-associated post-translational modification in S. cerevisiae." Nature cell biology **12**: 294-298.

Banerjee, T. and D. Chakravarti (2011). "A peek into the complex realm of histone phosphorylation." Molecular and cellular biology **31**: 4858-4873.

Banks, D. D. and L. M. Gloss (2004). "Folding mechanism of the (H3 – H4) 2 histone tetramer of the core nucleosome." Protein science **13**: 1304-1316.

Bannister, A. J., P. Zegerman, J. F. Partridge, E. A. Miska, J. O. Thomas, R. C. Allshire and T. Kouzarides (2001). "Selective recognition of methylated lysine 9 on histone H3 by the HP1 chromo domain." Nature **410**(6824): 120-124.

Batta, K., Z. Zhang, K. Yen, D. B. Goffman and B. F. Pugh (2011). "Genome-wide function of H2B ubiquitylation in promoter and genic regions." Genes & Development **25**: 2254-2265.

Bauer, W. R., J. J. Hayes, J. H. White and a. P. Wolffe (1994). Nucleosome structural changes due to acetylation. Journal of molecular biology. **236:** 685-690.

Belotserkovskaya, R., S. Oh, V. A. Bondarenko, G. Orphanides, V. M. Studitsky and D. Reinberg (2003). "FACT facilitates transcription-dependent nucleosome alteration." Science **301**(5636): 1090-1093.

Belotserkovskaya, R. and D. Reinberg (2004). "Facts about FACT and transcript elongation through chromatin." Current opinion in genetics & development **14**: 139-146.

Bode, J., M. M. Gomezlira and H. Schroter (1983). "Nucleosomal Particles Open as the Histone Core Becomes Hyperacetylated." European Journal of Biochemistry **130**(3): 437-445.

Bode, J., K. Henco and E. Wingender (1980). "Modulation of the nucleosome structure by histone acetylation." Eur J Biochem **110**(1): 143-152.

Böhm, V., A. R. Hieb, A. J. Andrews, A. Gansen, A. Rocker, K. Tóth, K. Luger and J. Langowski (2011). "Nucleosome accessibility governed by the dimer/tetramer interface." Nucleic acids research **39**: 3093-3102.

Bondarenko, V. A., L. M. Steele, A. Ujvari, D. A. Gaykalova, O. I. Kulaeva, Y. S. Polikanov, D. S. Luse and V. M. Studitsky (2006). "Nucleosomes can form a polar barrier to transcript elongation by RNA polymerase II." Mol Cell **24**(3): 469-479.

Brehove, M., T. Wang, J. North, Y. Luo, S. J. Dreher, J. C. Shimko, J. J. Ottesen, K. Luger and M. G. Poirier (2015). "Histone Core Phosphorylation Regulates DNA Accessibility." J Biol Chem.

Brookes, E., W. Cao and B. Demeler (2010). "A two-dimensional spectrum analysis for sedimentation velocity experiments of mixtures with heterogeneity in molecular weight and shape." Eur Biophys J **39**(3): 405-414.

Brookes, E. H. and B. Demeler (2007). "Parsimonious Regularization using Genetic Algorithms Applied to the Analysis of Analytical Ultracentrifugation Experiments." Gecco 2007: Genetic and Evolutionary Computation Conference, Vol 1 and 2: 361-368.

Burgess, R. J. and Z. Zhang (2013). "Histone chaperones in nucleosome assembly and human disease." Nature structural & molecular biology **20**: 14-22.

Byvoet, P. and G. Shepherd (1972). "The distribution and turnover of labeled methyl groups in histone fractions of cultured mammalian cells." Archives of biochemistry and biophysics **148**: 558-567.

Carvalho, S., A. C. Raposo, F. B. Martins, A. R. Grosso, S. C. Sridhara, J. Rino, M. Carmo-Fonseca and S. F. de Almeida (2013). Histone methyltransferase SETD2 coordinates FACT recruitment with nucleosome dynamics during transcription. Nucleic acids research. **41:** 2881-2893.

Casadio, F., X. Lu, S. B. Pollock, G. LeRoy, B. a. Garcia, T. W. Muir, R. G. Roeder and C. D. Allis (2013). "H3R42me2a is a histone modification with positive transcriptional effects." Proceedings of the National Academy of Sciences of the United States of America **110**: 14894-14899.

Chen, A., F. E. Kleiman, J. L. Manley, T. Ouchi and Z. Q. Pan (2002). "Autoubiquitination of the BRCA1*BARD1 RING ubiquitin ligase." J Biol Chem **277**(24): 22085-22092.

Chen, C. C., J. J. Carson, J. Feser, B. Tamburini, S. Zabaronick, J. Linger and J. K. Tyler (2008). "Acetylated lysine 56 on histone H3 drives chromatin assembly after repair and signals for the completion of repair." Cell **134**: 231-243.

Cosgrove, M. S., J. D. Boeke and C. Wolberger (2004). "Regulated nucleosome mobility and the histone code." Nature structural & molecular biology **11**: 1037-1043.

Daujat, S., T. Weiss, F. Mohn, U. C. Lange, C. Ziegler-Birling, U. Zeissler, M. Lappe, D. Schübeler, M.-E. Torres-Padilla and R. Schneider (2009). "H3K64 trimethylation marks heterochromatin and is dynamically remodeled during developmental reprogramming." Nature structural & molecular biology **16**: 777-781.

Dawson, M. a., A. J. Bannister, B. Göttgens, S. D. Foster, T. Bartke, A. R. Green and T. Kouzarides (2009). "JAK2 phosphorylates histone H3Y41 and excludes HP1alpha from chromatin." Nature **461**: 819-822.

Dawson, M. a., S. D. Foster, A. J. Bannister, S. C. Robson, R. Hannah, X. Wang, B. Xhemalce, A. D. Wood, A. R. Green, B. Göttgens and T. Kouzarides (2012). "Three distinct patterns of histone H3Y41 phosphorylation mark active genes." Cell reports **2**: 470-477.

Demeler, B. and E. Brookes (2008). "Monte Carlo analysis of sedimentation experiments." Colloid and Polymer Science **286**(2): 129-137.

Demeler, B. and K. E. van Holde (2004). "Sedimentation velocity analysis of highly heterogeneous systems." Anal Biochem **335**(2): 279-288.

Dion, M. F., T. Kaplan, M. Kim, S. Buratowski, N. Friedman and O. J. Rando (2007). "Dynamics of replication-independent histone turnover in budding yeast." Science **315**(5817): 1405-1408.

Donham, D. C., J. K. Scorgie and M. E. A. Churchill (2011). "The activity of the histone chaperone yeast Asf1 in the assembly and disassembly of histone H3/H4–DNA complexes." Nucleic Acids Research **39**(13): 5449-5458.

Driscoll, R., A. Hudson and S. P. Jackson (2007). "Yeast Rtt109 promotes genome stability by acetylating histone H3 on lysine 56." Science **315**(5812): 649-652.

Dyer, P. N., R. S. Edayathumangalam, C. L. White, Y. Bao, S. Chakravarthy, U. M. Muthurajan and K. Luger (2004). "Reconstitution of nucleosome core particles from recombinant histones and DNA." Methods Enzymol **375**: 23-44.

Eitoku, M., L. Sato, T. Senda and M. Horikoshi (2008). "Histone chaperones: 30 years from isolation to elucidation of the mechanisms of nucleosome assembly and disassembly." Cell Mol Life Sci **65**(3): 414-444.

Emre, N. C., K. Ingvarsdottir, A. Wyce, A. Wood, N. J. Krogan, K. W. Henry, K. Li, R. Marmorstein, J. F. Greenblatt, A. Shilatifard and S. L. Berger (2005). "Maintenance of low histone ubiquitylation by Ubp10 correlates with telomere-proximal Sir2 association and gene silencing." Mol Cell **17**(4): 585-594.

English, C. M., M. W. Adkins, J. J. Carson, M. E. Churchill and J. K. Tyler (2006). "Structural basis for the histone chaperone activity of Asf1." Cell **127**(3): 495-508.

Fang, H., D. J. Clark and J. J. Hayes (2011). "DNA and nucleosomes direct distinct folding of a linker histone H1 C-terminal domain." Nucleic acids research: 1-10.

Ferreira, H., J. Somers, R. Webster, A. Flaus and T. Owen-Hughes (2007). "Histone tails and the H3 alphaN helix regulate nucleosome mobility and stability." Molecular and cellular biology **27**: 4037-4048.

Fischle, W., Y. Wang and C. D. Allis (2003). "Histone and chromatin cross-talk." Curr Opin Cell Biol **15**(2): 172-183.

Flaus, A., K. Luger, S. Tan and T. J. Richmond (1996). "Mapping nucleosome position at single base-pair resolution by using site-directed hydroxyl radicals." Proceedings of the National Academy of Sciences of the United States of America **93**(4): 1370-1375.

Floer, M., X. Wang, V. Prabhu, G. Berrozpe, S. Narayan, D. Spagna, D. Alvarez, J. Kendall, A. Krasnitz, A. Stepansky, J. Hicks, G. O. Bryant and M. Ptashne (2010). "A RSC/nucleosome complex determines chromatin architecture and facilitates activator binding." Cell **141**(3): 407-418.

Formosa, T., P. Eriksson, J. Wittmeyer, J. Ginn, Y. Yu and D. J. Stillman (2001). Spt16-Pob3 and the HMG protein Nhp6 combine to form the nucleosome-binding factor SPN. The EMBO journal. **20:** 3506-3517.

Fujii-Nakata, T., Y. Ishimi, A. Okuda and A. Kikuchi (1992). "Functional analysis of nucleosome assembly protein, NAP-1. The negatively charged COOH-terminal region is not necessary for the intrinsic assembly activity." J Biol Chem **267**(29): 20980-20986.

Goldknopf, I. L., C. W. Taylor, R. M. Baum, L. C. Yeoman, M. O. Olson, A. W. Prestayko and H. Busch (1975). "Isolation and characterization of protein A24, a "histone-like" non-histone chromosomal protein." J Biol Chem **250**(18): 7182-7187.

Goldman, J. A., J. D. Garlick and R. E. Kingston (2010). "Chromatin remodeling by imitation switch (ISWI) class ATP-dependent remodelers is stimulated by histone variant H2A.Z." J. Biol. Chem. **285**: 4645-4651.

Goldstein, G., M. Scheid, U. Hammerling, D. H. Schlesinger, H. D. Niall and E. A. Boyse (1975). "Isolation of a polypeptide that has lymphocyte-differentiating properties and is probably represented universally in living cells." Proceedings of the National Academy of Sciences of the United States of America **72**(1): 11-15.

Hall, M. A., A. Shundrovsky, L. Bai, R. M. Fulbright, J. T. Lis and M. D. Wang (2009). "High-resolution dynamic mapping of histone-DNA interactions in a nucleosome." Nat Struct Mol Biol **16**(2): 124-129.

Henry, K. W., A. Wyce, W. S. Lo, L. J. Duggan, N. C. Emre, C. F. Kao, L. Pillus, A. Shilatifard, M. A. Osley and S. L. Berger (2003). "Transcriptional activation via sequential histone H2B ubiquitylation and deubiquitylation, mediated by SAGA-associated Ubp8." Genes Dev **17**(21): 2648-2663.

Hieb, A. R., S. D'Arcy, M. A. Kramer, A. E. White and K. Luger (2012). "Fluorescence

strategies for high-throughput quantification of protein interactions." Nucleic Acids Res. **40**: e33.

Hondele, M., T. Stuwe, M. Hassler, F. Halbach, A. Bowman, E. T. Zhang, B. Nijmeijer, C. Kotthoff, V. Rybin, S. Amlacher, E. Hurt and A. G. Ladurner (2013). Structural basis of histone H2A-H2B recognition by the essential chaperone FACT. Nature.

Howman, E. V. (2000). "Early disruption of centromeric chromatin organization in centromere protein A (Cenpa) null mice." Proc. Natl Acad. Sci. USA **97**: 1148-1153.

Hsieh, F.-K., O. I. Kulaeva, S. S. Patel, P. N. Dyer, K. Luger, D. Reinberg and V. M. Studitsky (2013). Histone chaperone FACT action during transcription through chromatin by RNA polymerase II. Proceedings of the National Academy of Sciences of the United States of America.

Hsieh, F. K., M. Fisher, A. Ujvari, V. M. Studitsky and D. S. Luse (2010). "Histone Sin mutations promote nucleosome traversal and histone displacement by RNA polymerase II." EMBO Rep **11**(9): 705-710.

Huang, J.-Y., W.-H. Chen, Y.-L. Chang, H.-T. Wang, W.-t. Chuang and S.-C. Lee (2006). "Modulation of nucleosome-binding activity of FACT by poly(ADP-ribosyl)ation." Nucleic acids research **34**: 2398-2407.

Huang, S., H. Zhou, D. Katzmann, M. Hochstrasser, E. Atanasova and Z. Zhang (2005). "Rtt106p is a histone chaperone involved in heterochromatin-mediated silencing." Proc Natl Acad Sci U S A **102**(38): 13410-13415.

Hyland, E. M., H. Molina, K. Poorey, C. Jie, Z. Xie, J. Dai, J. Qian, S. Bekiranov, D. T. Auble, A. Pandey and J. D. Boeke (2011). "An evolutionarily 'young' lysine residue in histone H3 attenuates transcriptional output in Saccharomyces cerevisiae." Genes Dev **25**(12): 1306-1319.

Hyland, E. M. E. M., H. Molina, K. Poorey, C. Jie, Z. Xie, J. Dai, J. Qian, S. Bekiranov, D. T. D. T. Auble, A. Pandey, others and J. D. Boeke (2011). "An evolutionarily‘young’ lysine residue in histone H3 attenuates transcriptional output in Saccharomyces cerevisiae." Genes & Development **25**: 1306-1319.

Ikura, T., S. Tashiro, A. Kakino, H. Shima, N. Jacob, R. Amunugama, K. Yoder, S. Izumi, I. Kuraoka, K. Tanaka, H. Kimura, M. Ikura, S. Nishikubo, T. Ito, A. Muto, K. Miyagawa, S. Takeda, R. Fishel, K. Igarashi and K. Kamiya (2007). "DNA damage-dependent acetylation and ubiquitination of H2AX enhances chromatin dynamics." Molecular and cellular biology **27**: 7028-7040.

Izban, M. G. and D. S. Luse (1991). "Transcription on nucleosomal templates by RNA polymerase II in vitro: inhibition of elongation with enhancement of sequence-specific pausing." Genes Dev **5**(4): 683-696.

Izzo, A., K. Kamieniarz-Gdula, F. Ramirez, N. Noureen, J. Kind, T. Manke, B. van Steensel and R. Schneider (2013). "The genomic landscape of the somatic linker histone subtypes

H1.1 to H1.5 in human cells." Cell Rep **3**(6): 2142-2154.

Jacobs, S. A., S. D. Taverna, Y. Zhang, S. D. Briggs, J. Li, J. C. Eissenberg, C. D. Allis and S. Khorasanizadeh (2001). "Specificity of the HP1 chromo domain for the methylated N-terminus of histone H3." Embo j **20**(18): 5232-5241.

Jamai, A., R. M. Imoberdorf and M. Strubin (2007). "Continuous histone H2B and transcription-dependent histone H3 exchange in yeast cells outside of replication." Mol Cell **25**(3): 345-355.

Jamai, A., A. Puglisi and M. Strubin (2009). "Histone Chaperone Spt16 Promotes Redeposition of the Original H3-H4 Histones Evicted by Elongating RNA Polymerase." Molecular cell **35**: 377-383.

Jessulat, M., M. Alamgir, H. Salsali, J. Greenblatt, J. Xu and A. Golshani (2008). "Interacting proteins Rtt109 and Vps75 affect the efficiency of non-homologous end-joining in Saccharomyces cerevisiae." Archives of biochemistry and biophysics **469**: 157-164.

Joo, H. Y., L. Zhai, C. Yang, S. Nie, H. Erdjument-Bromage, P. Tempst, C. Chang and H. Wang (2007). "Regulation of cell cycle progression and gene expression by H2A deubiquitination." Nature **449**(7165): 1068-1072.

Jørgensen, S., G. Schotta and C. S. Sørensen (2013). "Histone H4 Lysine 20 methylation: key player in epigenetic regulation of genomic integrity." Nucleic acids research: 1-10.

Kadyrova, L. Y., E. Rodriges Blanko and F. A. Kadyrov (2013). "Human CAF-1-dependent nucleosome assembly in a defined system." Cell Cycle **12**(20): 3286-3297.

Kadyrova, L. Y., E. Rodriges Blanko and F. a. Kadyrov (2013). "Human CAF-1-dependent nucleosome assembly in a defined system." Cell cycle (Georgetown, Tex.) **12**: 3286-3297.

Kalashnikova, A. a., D. D. Winkler, S. J. McBryant, R. K. Henderson, J. a. Herman, J. G. Deluca, K. Luger, J. E. Prenni and J. C. Hansen (2013). Linker histone H1.0 interacts with an extensive network of proteins found in the nucleolus. Nucleic acids research.

Kaplan, C. D., L. Laprade and F. Winston (2003). "Transcription elongation factors repress transcription initiation from cryptic sites." Science **301**(5636): 1096-1099.

Keller, D. M. and H. Lu (2002). "p53 serine 392 phosphorylation increases after UV through induction of the assembly of the CK2.hSPT16.SSRP1 complex." J Biol Chem **277**(51): 50206-50213.

Kelly, W. G. and G. W. Hart (1989). "Glycosylation of chromosomal proteins: localization of O-linked N-acetylglucosamine in Drosophila chromatin." Cell **57**: 243-251.

Kemble, D. J., L. L. McCullough, F. G. Whitby, T. Formosa and C. P. Hill (2015). "FACT Disrupts Nucleosome Structure by Binding H2A-H2B with Conserved Peptide Motifs." Mol Cell.

Kemble, D. J., F. G. Whitby, H. Robinson, L. L. McCullough, T. Formosa and C. P. Hill (2013). Structure of the Spt16 middle domain reveals functional features of the histone chaperone FACT. The Journal of biological chemistry. **288:** 10188-10194.

Konarev, P. V., V. V. Volkov, A. V. Sokolova, M. H. J. Koch and D. I. Svergun (2003). "PRIMUS: a Windows PC-based system for small-angle scattering data analysis." Journal of Applied Crystallography **36**(5): 1277-1282.

Koopmans, W. J. A., A. Brehm, C. Logie, T. Schmidt and J. van Noort (2007). "Single-Pair FRET Microscopy Reveals Mononucleosome Dynamics." Journal of Fluorescence **17**(6): 785-795.

Kozin, M. B. and D. I. Svergun (2001). "Automated matching of high- and low-resolution structural models." Journal of Applied Crystallography **34**(1): 33-41.

Kulaeva, O. I., D. A. Gaykalova and V. M. Studitsky (2007). "Transcription through chromatin by RNA polymerase II: histone displacement and exchange." Mutat Res **618**(1-2): 116-129.

Kulaeva, O. I., F. K. Hsieh, H. W. Chang, D. S. Luse and V. M. Studitsky (2013). "Mechanism of transcription through a nucleosome by RNA polymerase II." Biochim Biophys Acta **1829**(1): 76-83.

Kuryan, B. G., J. Kim, N. N. H. Tran, S. R. Lombardo, S. Venkatesh, J. L. Workman and M. Carey (2012). "Histone density is maintained during transcription mediated by the chromatin remodeler RSC and histone chaperone NAP1 in vitro." Proceedings of the National Academy of Sciences of the United States of America **109**: 1931-1936.

Larschan, E., A. a. Alekseyenko, A. a. Gortchakov, S. Peng, B. Li, P. Yang, J. L. Workman, P. J. Park and M. I. Kuroda (2007). "MSL complex is attracted to genes marked by H3K36 trimethylation using a sequence-independent mechanism." Molecular cell **28**: 121-133.

Laskey, R. A., B. M. Honda, A. D. Mills and J. T. Finch (1978). "Nucleosomes are assembled by an acidic protein which binds histones and transfers them to DNA." Nature **275**(5679): 416-420.

Lee, Y.-H., S. a. Coonrod, W. L. Kraus, M. A. Jelinek and M. R. Stallcup (2005). "Regulation of coactivator complex assembly and function by protein arginine methylation and demethylimination." Proceedings of the National Academy of Sciences of the United States of America **102**: 3611-3616.

Leman, A. R. and E. Noguchi (2013). "The Replication Fork: Understanding the Eukaryotic Replication Machinery and the Challenges to Genome Duplication." Genes **4**(1): 1-32.

Levchenko, V. and V. Jackson (2004). "Histone release during transcription: NAP1 forms a complex with H2A and H2B and facilitates a topologically dependent release of H3 and H4 from the nucleosome." Biochemistry **43**(9): 2359-2372.

Li, G. and D. Reinberg (2011). "Chromatin higher-order structures and gene regulation."

October **21**: 175-186.

Li, Y., D. M. Keller, J. D. Scott and H. Lu (2005). "CK2 phosphorylates SSRP1 and inhibits its DNA-binding activity." The Journal of biological chemistry **280**: 11869-11875.

Long, L., M. Furgason and T. Yao (2014). "Generation of nonhydrolyzable ubiquitin–histone mimics." Methods **70**(2–3): 134-138.

Lu, X., M. D. Simon, J. V. Chodaparambil, J. C. Hansen, K. M. Shokat and K. Luger (2008). "The effect of H3K79 dimethylation and H4K20 trimethylation on nucleosome and chromatin structure." Nature structural & molecular biology **15**: 1122-1124.

Luebben, W. R., N. Sharma and J. K. Nyborg (2010). "Nucleosome eviction and activated transcription require p300 acetylation of histone H3 lysine 14." Proceedings of the National Academy of Sciences of the United States of America **107**: 19254-19259.

Luger, K., A. Mader, R. Richmond and D. Sargent (1997). "Crystal structure of the nucleosome core particle at 2.8 A resolution." Nature **389**: 251-260.

Luger, K., A. W. Mader, R. K. Richmond, D. F. Sargent and T. J. Richmond (1997). "Crystal structure of the nucleosome core particle at 2.8 A resolution." Nature **389**: 251-260.

Luger, K., T. J. Rechsteiner and T. J. Richmond (1999). "Expression and purification of recombinant histones and nucleosome reconstitution." Methods Mol Biol **119**: 1-16.

Manohar, M., A. M. Mooney, J. A. North, R. J. Nakkula, J. W. Picking, A. Edon, R. Fishel, M. G. Poirier and J. J. Ottesen (2009). "Acetylation of histone H3 at the nucleosome dyad alters DNA-histone binding." J Biol Chem **284**(35): 23312-23321.

Manohar, M., A. M. Mooney, J. a. North, R. J. Nakkula, J. W. Picking, A. Edon, R. Fishel, M. G. Poirier and J. J. Ottesen (2009). "Acetylation of histone H3 at the nucleosome dyad alters DNA-histone binding." The Journal of biological chemistry **284**: 23312-23321.

Marciano, G. and D. T. Huang (2016). "Structure of the human histone chaperone FACT Spt16 N-terminal domain." Acta Crystallogr F Struct Biol Commun **72**(Pt 2): 121-128.

Masumoto, H., D. Hawke, R. Kobayashi and A. Verreault (2005). "A role for cell-cycle-regulated histone H3 lysine 56 acetylation in the DNA damage response." Nature **436**: 294-298.

McBryant, S. J., Y. J. Park, S. M. Abernathy, P. J. Laybourn, J. K. Nyborg and K. Luger (2003). "Preferential binding of the histone (H3-H4)2 tetramer by NAP1 is mediated by the amino-terminal histone tails." J Biol Chem **278**(45): 44574-44583.

McCullough, L., B. Poe, Z. Connell, H. Xin and T. Formosa (2013). "The FACT histone chaperone guides histone H4 into its nucleosomal conformation in Saccharomyces cerevisiae." Genetics **195**: 101-113.

Minsky, N., E. Shema, Y. Field, M. Schuster, E. Segal and M. Oren (2008). "Monoubiquitinated H2B is associated with the transcribed region of highly expressed

genes in human cells." Nat Cell Biol **10**(4): 483-488.

Mirsky, a. E., B. Silverman and N. C. Panda (1972). "Blocking by histones of accessibility to DNA in chromatin: addition of histones." Proceedings of the National Academy of Sciences of the United States of America **69**: 3243-3246.

Molina-Serrano, D. and A. Kirmizis (2013). Beyond the histone tail: Acetylation at the nucleosome dyad commands transcription. Nucleus (Austin, Tex.). **4**.

Morgan, M. T., M. Haj-Yahya, A. E. Ringel, P. Bandi, A. Brik and C. Wolberger (2016). "Structural basis for histone H2B deubiquitination by the SAGA DUB module." Science **351**(6274): 725-728.

Nakagawa, T., T. Kajitani, S. Togo, N. Masuko, H. Ohdan, Y. Hishikawa, T. Koji, T. Matsuyama, T. Ikura, M. Muramatsu and T. Ito (2008). "Deubiquitylation of histone H2A activates transcriptional initiation via trans-histone cross-talk with H3K4 di- and trimethylation." Genes Dev **22**(1): 37-49.

Neumann, H., S. M. Hancock, R. Buning, A. Routh, L. Chapman, J. Somers, T. Owen-Hughes, J. van Noort, D. Rhodes and J. W. Chin (2009). "A method for genetically installing site-specific acetylation in recombinant histones defines the effects of H3 K56 acetylation." Molecular cell **36**: 153-163.

Nguyen, H.-T. T., W. Wharton, J. a. Harper, J. R. Dornhoffer and A. a. Duina (2013). A Nucleosomal Region Important for Ensuring Proper Interactions Between the Transcription Elongation Factor Spt16 and Transcribed Genes in Saccharomyces cerevisiae. G3 (Bethesda, Md.). **3:** 929-940.

Nishino, Y., M. Eltsov, Y. Joti, K. Ito, H. Takata, Y. Takahashi, S. Hihara, A. S. Frangakis, N. Imamoto, T. Ishikawa and K. Maeshima (2012). "Human mitotic chromosomes consist predominantly of irregularly folded nucleosome fibres without a 30-nm chromatin structure." The EMBO journal: 1-10.

Orphanides, G., G. LeRoy, C. H. Chang, D. S. Luse and D. Reinberg (1998). FACT, a factor that facilitates transcript elongation through nucleosomes. Cell. **92:** 105-116.

Orphanides, G., W. H. Wu, W. S. Lane, M. Hampsey and D. Reinberg (1999). "The chromatin-specific transcription elongation factor FACT comprises human SPT16 and SSRP1 proteins." Nature **400**(6741): 284-288.

Orphanides, G., W. H. Wu, W. S. Lane, M. Hampsey and D. Reinberg (1999). "The chromatin-specific transcription elongation factor FACT comprises human SPT16 and SSRP1 proteins." Nature **400**: 284-288.

Pavri, R., B. Zhu, G. Li, P. Trojer, S. Mandal, A. Shilatifard and D. Reinberg (2006). "Histone H2B monoubiquitination functions cooperatively with FACT to regulate elongation by RNA polymerase II." Cell **125**(4): 703-717.

Perez-Lamigueiro, M. A. and R. Alvarez-Gonzalez (2004). "Polynucleosomal synthesis of poly(ADP-ribose) causes chromatin unfolding as determined by micrococcal nuclease

digestion." Ann N Y Acad Sci **1030**: 593-598.

Phillips, D. M. (1963). "The presence of acetyl groups of histones." Biochem J **87**: 258-263.

Poirier, M. G., M. Bussiek, J. Langowshi and J. Widom (2008). "Spontaneous access to DNA target sites in folded chromatin fibers." Brain, Behavior, and Immunity **22**: 629.

Poirier, M. G., M. Bussiek, J. Langowski and J. Widom (2008). "Spontaneous access to DNA target sites in folded chromatin fibers." J. Mol. Biol. **379**: 772-786.

Potoyan, D. a. and G. a. Papoian (2012). "Regulation of the H4 tail binding and folding landscapes via Lys-16 acetylation." Proceedings of the National Academy of Sciences of the United States of America **109**: 17857-17862.

Price, B. D. and A. D. D'Andrea (2013). "Chromatin Remodeling at DNA Double Strand Breaks." Cell **152**(6): 1344-1354.

Ramachandran, S., L. Vogel, B. D. Strahl and N. V. Dokholyan (2011). "Thermodynamic stability of histone H3 is a necessary but not sufficient driving force for its evolutionary conservation." PLoS Comput Biol **7**(1): e1001042.

Rhoades, A. R., S. Ruone and T. Formosa (2004). "Structural Features of Nucleosomes Reorganized by Yeast FACT and Its HMG Box Component, Nhp6." Molecular and Cellular Biology **24**(9): 3907-3917.

Robinson, P. J. and D. Rhodes (2006). "Structure of the '30 nm' chromatin fibre: a key role for the linker histone." Curr Opin Struct Biol **16**(3): 336-343.

Robzyk, K., J. Recht and M. A. Osley (2000). "Rad6-dependent ubiquitination of histone H2B in yeast." Science **287**(5452): 501-504.

Ruone, S., A. R. Rhoades and T. Formosa (2003). "Multiple Nhp6 molecules are required to recruit Spt16-Pob3 to form yFACT complexes and to reorganize nucleosomes." J Biol Chem **278**(46): 45288-45295.

Santisteban, M. S., G. Arents, E. N. Moudrianakis and M. M. Smith (1997). "Histone octamer function in vivo: mutations in the dimer-tetramer interfaces disrupt both gene activation and repression." Embo j **16**(9): 2493-2506.

Scheffner, M., U. Nuber and J. M. Huibregtse (1995). "Protein ubiquitination involving an E1-E2-E3 enzyme ubiquitin thioester cascade." Nature **373**: 81-83.

Scheuermann, J. C., A. G. de Ayala Alonso, K. Oktaba, N. Ly-Hartig, R. K. McGinty, S. Fraterman, M. Wilm, T. W. Muir and J. Muller (2010). "Histone H2A deubiquitinase activity of the Polycomb repressive complex PR-DUB." Nature **465**(7295): 243-247.

Schlesinger, M. B. and T. Formosa (2000). "POB3 is required for both transcription and replication in the yeast Saccharomyces cerevisiae." Genetics **155**(4): 1593-1606.

Shepherd, G., J. Hardin and B. Noland (1971). "Methylation of lysine residues of histone

fractions in synchronized mommalian cells." Archives of biochemistry and biophysics **143**: 1-5.

Shiio, Y. and R. N. Eisenman (2003). "Histone sumoylation is associated with transcriptional repression." Proceedings of the National Academy of Sciences of the United States of America **100**: 13225-13230.

Shilatifard, A. (2006). "Chromatin modifications by methylation and ubiquitination: implications in the regulation of gene expression." Annual review of biochemistry **75**: 243-269.

Siman, P., S. V. Karthikeyan, M. Nikolov, W. Fischle and A. Brik (2013). "Convergent Chemical Synthesis of Histone H2B Protein for the Site-Specific Ubiquitination at Lys34." Angewandte Chemie (International ed. in English) **52**: 8059-8063.

Simon, M., J. a. North, J. C. Shimko, R. a. Forties, M. B. Ferdinand, M. Manohar, M. Zhang, R. Fishel, J. J. Ottesen and M. G. Poirier (2011). "Histone fold modifications control nucleosome unwrapping and disassembly." Proceedings of the National Academy of Sciences **108**: 12711-12716.

Simon, M. D., F. Chu, L. R. Racki, C. C. de la Cruz, A. L. Burlingame, B. Panning, G. J. Narlikar and K. M. Shokat (2007). "The site-specific installation of methyl-lysine analogs into recombinant histones." Cell **128**(5): 1003-1012.

Singer, R. A. and G. C. Johnston (2004). "The FACT chromatin modulator: genetic and structure/function relationships." Biochem Cell Biol **82**(4): 419-427.

Smith, S. and B. Stillman (1991). "Stepwise assembly of chromatin during DNA replication in vitro." EMBO **10**: 971-980.

Stillman, B. (1986). "Chromatin assembly during SV40 DNA replication in vitro." Cell **45**(4): 555-565.

Stillman, B. W. and Y. Gluzman (1985). "Replication and supercoiling of simian virus 40 DNA in cell extracts from human cells." Mol Cell Biol **5**(8): 2051-2060.

Stillman, D. J. (2010). "Nhp6: A small but powerful effector of chromatin structure in Saccharomyces cerevisiae." Biochimica et Biophysica Acta (BBA) - Gene Regulatory Mechanisms **1799**(1–2): 175-180.

Stoler, S., K. C. Keith, K. E. Curnick and M. Fitzgerald-Hayes (1995). "A mutation in CSE4, an essential gene encoding a novel chromatin-associated protein in yeast, causes chromosome nondisjunction and cell cycle arrest at mitosis." Genes Dev. **9**: 573-586.

Strahl, B. D. and C. D. Allis (2000). "The language of covalent histone modifications." Nature **403**: 41-45.

Stutzer, A., S. Liokatis, A. Kiesel, D. Schwarzer, R. Sprangers, J. Soding, P. Selenko and W. Fischle (2016). "Modulations of DNA Contacts by Linker Histones and Post-translational Modifications Determine the Mobility and Modifiability of Nucleosomal H3

Tails." Mol Cell **61**(2): 247-259.

Svergun, D. I., L. Feigin and G. W. Taylor (1987). "Structure analysis by small angle X-ray and neutron scattering."

Svergun, D. I. and M. H. J. Koch (2003). "Small-angle scattering studies of biological macromolecules in solution." Reports on Progress in Physics **66**: 1735.

Takahata, S., Y. Yu and D. J. Stillman (2009). "The E2F functional analogue SBF recruits the Rpd3(L) HDAC, via Whi5 and Stb1, and the FACT chromatin reorganizer, to yeast G1 cyclin promoters." Embo j **28**(21): 3378-3389.

Thiriet, C. and J. J. Hayes (2005). "Replication-independent core histone dynamics at transcriptionally active loci in vivo." Genes Dev **19**(6): 677-682.

Thoma, F., T. Koller and A. Klug (1979). "Involvement of histone H1 in the organization of the nucleosome and of the salt-dependent superstructures of chromatin." J Cell Biol **83**(2 Pt 1): 403-427.

Thorne, A. W., P. Sautiere, G. Briand and C. Crane-Robinson (1987). "The structure of ubiquitinated histone H2B." Embo j **6**(4): 1005-1010.

Tropberger, P., S. Pott, C. Keller, K. Kamienizrz-Gdula, M. Caron, F. Richter, G. Li, G. Mittler, E. T.Liu, M. Buhler, R. Margueron and R. Schneider (2013). "Regulation of Transcription through Acetylation of H3K122 on the Lateral Surface of the Histone Octamer." Cell **152**: 859-872.

Tsunaka, Y., Y. Fujiwara, T. Oyama, S. Hirose and K. Morikawa (2016). "Integrated molecular mechanism directing nucleosome reorganization by human FACT." Genes Dev.

Tsunaka, Y., J. Toga, H. Yamaguchi, S. Tate, S. Hirose and K. Morikawa (2009). "Phosphorylated intrinsically disordered region of FACT masks its nucleosomal DNA binding elements." J Biol Chem **284**(36): 24610-24621.

Tweedie-Cullen, R. Y., J. M. Reck and I. M. Mansuy (2009). "Comprehensive mapping of post-translational modifications on synaptic, nuclear, and histone proteins in the adult mouse brain." J Proteome Res **8**(11): 4966-4982.

Tyler, J. K., C. R. Adams, S. R. Chen, R. Kobayashi, R. T. Kamakaka and J. T. Kadonaga (1999). "The RCAF complex mediates chromatin assembly during DNA replication and repair." Nature **402**(6761): 555-560.

Ueda, K., A. Omachi, M. Kawaichi and O. Hayaishi (1975). "Natural occurrence of poly(ADP-ribosyl) histones in rat liver." Proceedings of the National Academy of Sciences of the United States of America **72**: 205-209.

Ujvári, A., F.-K. Hsieh, S. W. Luse, V. M. Studitsky and D. S. Luse (2008). "Histone N-terminal tails interfere with nucleosome traversal by RNA polymerase II." The Journal of biological chemistry **283**: 32236-32243.

Unnikrishnan, A., P. R. Gafken and T. Tsukiyama (2010). "Dynamic changes in histone acetylation regulate origins of DNA replication." Nature structural & molecular biology **17**: 430-437.

Venkatesh, S., M. Smolle, H. Li and M. Gogol (2012). "Set2 methylation of histone H3 lysine 36 suppresses histone exchange on transcribed genes." Nature **000**: 1-4.

Voth, W. P., S. Takahata, J. L. Nishikawa, B. M. Metcalfe, A. M. Näär and D. J. Stillman (2014). "A Role for FACT in Repopulation of Nucleosomes at Inducible Genes." PloS one **9**: e84092.

Wang, H., L. Wang, H. Erdjument-bromage, M. Vidal, P. Tempst, R. S.Jones and Y. Zhang (2004). "Role of histone H2A ubiquitination in Polycomb silencing." Nature **431**: 873-878.

West, M. and W. Bonner (1980). "Histone 2B can be modified by the attachment of ubiquitin." Nucleic acids research **8**: 4671-4680.

White, A. E., A. R. Hieb and K. Luger (2016). "A quantitative investigation of linker histone interactions with nucleosomes and chromatin." Scientific Reports **6**: 19122.

Williams, S. K., D. Truong and J. K. Tyler (2008). "Acetylation in the globular core of histone H3 on lysine-56 promotes chromatin disassembly during transcriptional activation." Proceedings of the National Academy of Sciences of the United States of America **105**: 9000-9005.

Winkler, D. D., U. M. Muthurajan, A. R. Hieb and K. Luger (2011). "Histone chaperone FACT coordinates nucleosome interaction through multiple synergistic binding events." The Journal of biological chemistry **286**: 41883-41892.

Winkler, D. D., H. Zhou, M. A. Dar, Z. Zhang and K. Luger (2012). "Yeast CAF-1 assembles histone (H3-H4)2 tetramers prior to DNA deposition." Nucleic Acids Res **40**(20): 10139-10149.

Wittmeyer, J. and T. Formosa (1997). "The Saccharomyces cerevisiae DNA polymerase alpha catalytic subunit interacts with Cdc68/Spt16 and with Pob3, a protein similar to an HMG1-like protein." Molecular and Cellular Biology **17**(7): 4178-4190.

Wu, L., L. Li, B. Zhou, Z. Qin and Y. Dou (2014). "H2B Ubiquitylation Promotes RNA Pol II Processivity via PAF1 and pTEFb." molecular Cell.

Wu, L., B. M. Zee, Y. Wang, B. a. Garcia and Y. Dou (2011). "The RING finger protein MSL2 in the MOF complex is an E3 ubiquitin ligase for H2B K34 and is involved in crosstalk with H3 K4 and K79 methylation." Molecular cell **43**: 132-144.

Xin, H., S. Takahata, M. Blanksma, L. McCullough, D. J. Stillman and T. Formosa (2009). "yFACT induces global accessibility of nucleosomal DNA without H2A-H2B displacement." Molecular cell **35**: 365-376.

Yang, C., M. J. van der Woerd, U. M. Muthurajan, J. C. Hansen and K. Luger (2011). "Biophysical analysis and small-angle X-ray scattering-derived structures of MeCP2-

nucleosome complexes." Nucleic acids research **39**: 4122-4135.

Zhang, L., E. E. Eugeni, M. R. Parthun and M. A. Freitas (2003). "Identification of novel histone post-translational modifications by peptide mass fingerprinting." Chromosoma **112**(2): 77-86.

Zhang, L., H. Ma and B. F. Pugh (2011). "Stable and dynamic nucleosome states during a meiotic developmental process." Genome Res **21**(6): 875-884.

Zhang, Q., H. A. Giebler, M. K. Isaacson and J. K. Nyborg (2015). "Eviction of linker histone H1 by NAP-family histone chaperones enhances activated transcription." Epigenetics Chromatin **8**: 30.

Zhang, Y. (2003). "Transcriptional regulation by histone ubiquitination and deubiquitination." Genes Dev **17**(22): 2733-2740.

Zhou, J., J. Y. Fan, D. Rangasamy and D. J. Tremethick (2007). "The nucleosome surface regulates chromatin compaction and couples it with transcriptional repression." Nature structural & molecular biology **14**: 1070-1076.

Zhou, W., P. Zhu, J. Wang, G. Pascual, K. A. Ohgi, J. Lozach, C. K. Glass and M. G. Rosenfeld (2008). "Histone H2A Monoubiquitination Represses Transcription by Inhibiting RNA Polymerase II Transcriptional Elongation." Molecular Cell **29**(1): 69-80.

Zhu, Q., G. M. Pao, A. M. Huynh, H. Suh, N. Tonnu, P. M. Nederlof, F. H. Gage and I. M. Verma (2011). "BRCA1 tumour suppression occurs via heterochromatin-mediated silencing." Nature **477**(7363): 179-184.

APPENDIX I

THE ROLE OF FACT AND NAP1 IN REGULATING H1 DYNAMICS

I.1 Summary

Linker histone H1 facilitates chromatin compaction via physical interaction with the nucleosome, thereby serving as a barrier for the accessibility of genetic information. Putative linker H1 histone chaperones have been identified, such as Nap1 and FACT. However, the direct role of FACT and Nap1 in H1 dynamics is still not clear. Here, we find that both Nap1 and FACT bind H1 with low nanomolar affinity. Nap1 binds globular domain of H1, while FACT binds to C-terminal tail of H1. Our biochemical data suggest that neither Nap1 or FACT removes H1 from nucleosome in vitro.

I.2 Introduction

Linker H1 histone is composed of short N-terminal domain (~20 a.a.), a globular domain (~80 a.a.) and a long highly basic C-terminal tail (~100 a.a.). Linker H1 histone binds nucleosome with picomolar affinity, resulting in chromatin compaction (White et al. 2016). It has been shown that H1 occupancy is negatively associated with active transcription (Izzo et al. 2013). This indicates that removing linker histone is pivotal for DNA dependent processes. However, the question of how H1 is removed from nucleosome still needs to be addressed. Our previous work reported that FACT binds to H1 with ~35 nM affinity (Kalashnikova et al. 2013). However, it is still unknown if FACT regulates H1 dynamics. Similarly, hNap1 also physically interact with H1 in vitro, and resulting in displacing H1 from chromatin in the presence of other factors, such as activators and p300 in an in vitro

transcription system (Zhang et al. 2015). Thus, the direct role of Nap1 in H1 dynamics still needs to be elucidated.

Here, we reveal that hFACT binds to H1 with ~40 nM affinity, while hFACT binds H2A-H2B and H3-H4 with affinity ~15 nM. We find that FACT interaction with partial C-terminal tail of H1 (97-121 a.a.), that is also crucial for H1 binding nucleosome linker DNA. It indicates that FACT may play an important role in H1 displacement from nucleosome. However, our biochemical data suggest that FACT is unable to remove H1 from mononucleosome. Compared to FACT, Nap1 binds to H1 with ~5 nM affinity. The C-terminal tail is not required for this interaction. Similarly, Nap1 does not remove H1 from nucleosome. This indicates that other factors might be required in vivo that weaken the interaction between linker histone and the nucleosome.

I.3 Material and Methods

I.3.1 Reagents

Recombinant histones were expressed and purified as described previously (Luger et al. 1999). Fluorescence labeled linker H1 histone was prepared as described previously (White et al. 2016). Nucleosomes with 207-bp DNA were reconstituted by salt dialysis (Dyer et al. 2004). FACT was prepared as mentioned above, while Nap1 was prepared as published (McBryant et al. 2003).

I.3.2 Fluorescence-based plated assay

Fluorescence-based plate assay was performed in 150 mM NaCl, 20 mM Tris pH 7.0, 0.01% CHAPS, 0.01% NP40 and 1 mM TCEP. Fluorescence microplate was prepared

as published before (Winkler et al. 2011). Fluorescence labeled Mouse linker H1 histone were kept at 1 nM. hFACT or yNap1 was titrated from 1 nM to 500 nM. The reaction mixture was incubated in a 384-well microplate for 10 mins at room temperature, and then scanned by Typhoon 8600 variable mode fluorimager. Fluorescence signal was quantified by ImageQuant^{TM}. The data was analyzed and fit by Graphpad Prism.

I.3.3 In vitro gel-based assay

To determine if FACT or Nap1 removes H1 from nucleosome, 100 nM 207 nucleosome was mixed with 50 nM H1 first, and then either FACT or Nap1 (50 nM to 1.6 μM) was added into the reaction mixture. Nucleosome-H1 complex was analyzed on a 5% PAGE.

I.4 Results

I.4.1 The C-terminal tail of H1 is required for FACT-H1 interaction, but not for Nap1-H1 interaction.

The binding affinity of hFACT or yNap1 for linker H1 histone was determined using a fluorescence-based plate assay (Figure I.1 and I.2). We reveal that hFACT binds H1 with an affinity of 40 nM (Table I.1). This result is consistent with our previous work (Kalashnikova et al. 2013). In contrast, Nap1 binds to H1 with an affinity of ~3 nM (Table I.1). Next, we wanted to test which region of H1 is required for FACT or Nap1-H1 interaction. Since H1 C-terminal tail plays an important role in H1-nucleosome interaction, we asked if FACT or Nap1 binds this region, which may indicate their potential role in H1 dynamics. Two H1 truncations (Δ98-193 a.a. or Δ122-193 a.a.) were studied. FACT or Nap1 binding affinity for each H1 construct was determined as above (Figure I.1 and I.2). Notably, we find that H1 C-terminal tail is not required for Nap1-H1 interaction, but is

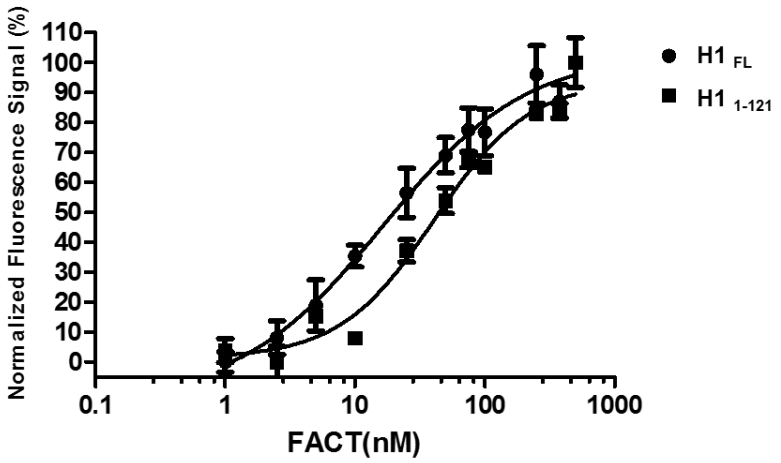


Figure I.1. The partial H1-CTD is crucial for FACT-H1 interaction. The binding affinity of FACT with different H1 construction was determined by fluorescence-based plate assay. H1 was labeled with Oregon Green. The concentration was kept at 1 nM. FACT was titrated from 1 nM to 500 nM. The reaction was performed under physiological salt condition (150 mM NaCl). Fluorescence signal is increased as a result of direct interaction between FACT and H1 $_{FL}$ or H1 $_{1\text{-}121}$. The binding affinity of FACT with H1 $_{1\text{-}97}$ was determined by the same approach. We found that FACT does not interact with H1 $_{1\text{-}97}$. It indicates that the partial C-terminal region (98 a.a.-121 a.a.) is essential for FACT interaction with H1. Each error bar represents SD of two biological replicates.

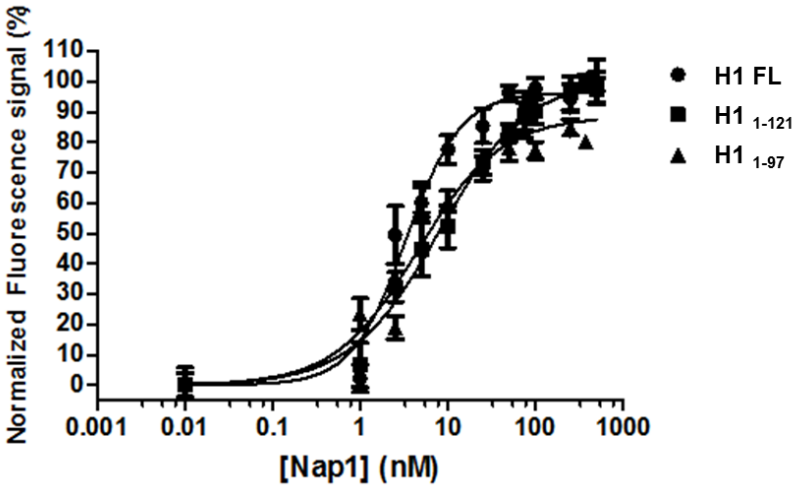


Figure I.2. H1-CTD is not required for Nap1-H1 interaction. The binding affinity of Nap1 with different H1 construction was determined as above. We found that Nap1 binds to all the H1 construction we designed. It indicates that C-terminal tail of H1 is not required for Nap1 interaction with H1. Each error bar represents SD of two biological replicates.

Table I.1 Summary for FACT-H1 and Nap1-H1 binding affinity

Protein	K_d	n	R^2
FACT-H1 $_{FL}$	39.96±6.34	2	0.96
FACT-H1 $_{1-121}$	16.36±6.78	2	0.88
FACT-H1 $_{1-97}$	No binding	2	
Nap1-H1 $_{FL}$	3.12±0.5	2	0.91
Nap1-H1 $_{1-121}$	6.97±1.62	2	0.93
Nap1-H1 $_{1-97}$	4.11±1.04	2	0.88

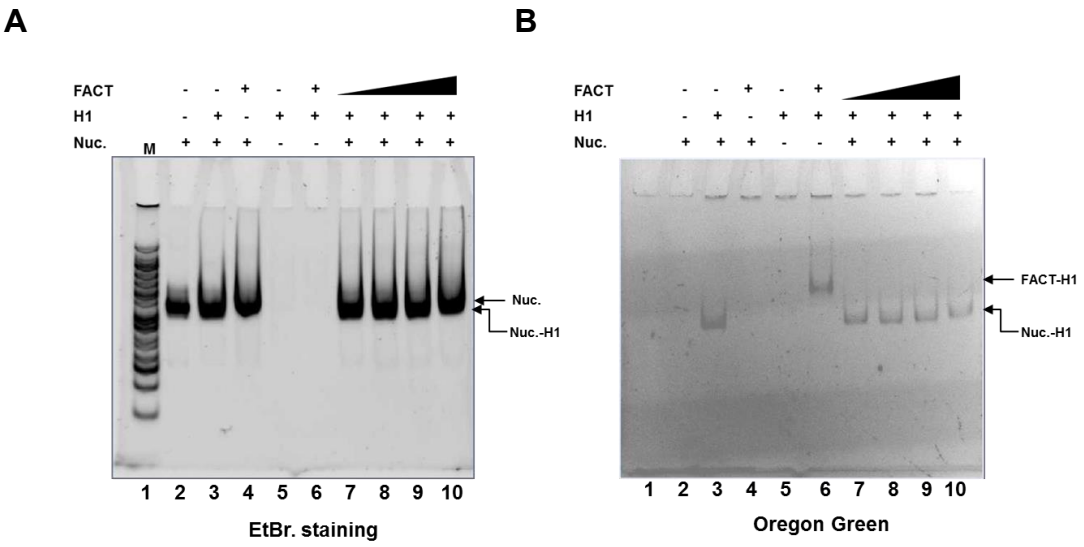
crucial for the FACT-H1 interaction. Specifically, the region between amino acids 93-121 is essential for FACT interaction with H1.

I.4.2 Neither FACT or Nap1 removes H1 from nucleosome.

In order to test if FACT or Nap1 facilitates H1 eviction from nucleosome, an in vitro gel-based assay was applied. 207 nucleosome was mixed with H1 first, and then either FACT or Nap1 was added. The complexes were analyzed on a 5% PAGE, and visualized by EtBr. staining or fluorescence (H1) (Figure I.3). As FACT or Nap1 is titrated, the amount of nucleosome-H1 complexes remains the same (Figure I.3 A-D lane 7-10), and neither FACT-H1 or Nap1-H1 complex was observed on the gel (Figure I.3 B and D lane 7-10). This indicates that either FACT or Nap1 does not remove H1 from nucleosome on its own.

I.5 Discussion

Linker H1 histone plays an important role in the accessibility of nucleosomal DNA. Thus, understanding how H1 dynamics is regulated is crucial to understand DNA dependent cellular processes. Here, we report that Nap1 and FACT bind to H1 with low nanomolar affinity. Additionally, we find that the C-terminal tail is essential for FACT-H1 interaction, but not for Nap1-H1 interaction. Subsequently, we investigated the role of FACT or Nap1 in H1 dynamics. Our biochemical data suggest that neither FACT nor Nap1 affects H1-nucleosome interaction. This is inconsistent with previous work (Zhang et al. 2015). It was suggested that hNap1 is able to facilitate H1 eviction from chromatin. This discrepancy may be due to different experimental condition. In previous work, hNap1 removal of H1 may be due to the presence of p300, which results in histone acetylation. Indeed, it has been shown that acetylation of H3 N-terminal tail reduces the occupancy of H1 on


A
B
FACT
H1
Nuc.
M
Nuc.
Nuc.-H1
1 2 3 4 5 6 7 8 9 10
EtBr. staining
FACT-H1
Nuc.-H1
1 2 3 4 5 6 7 8 9 10
Oregon Green

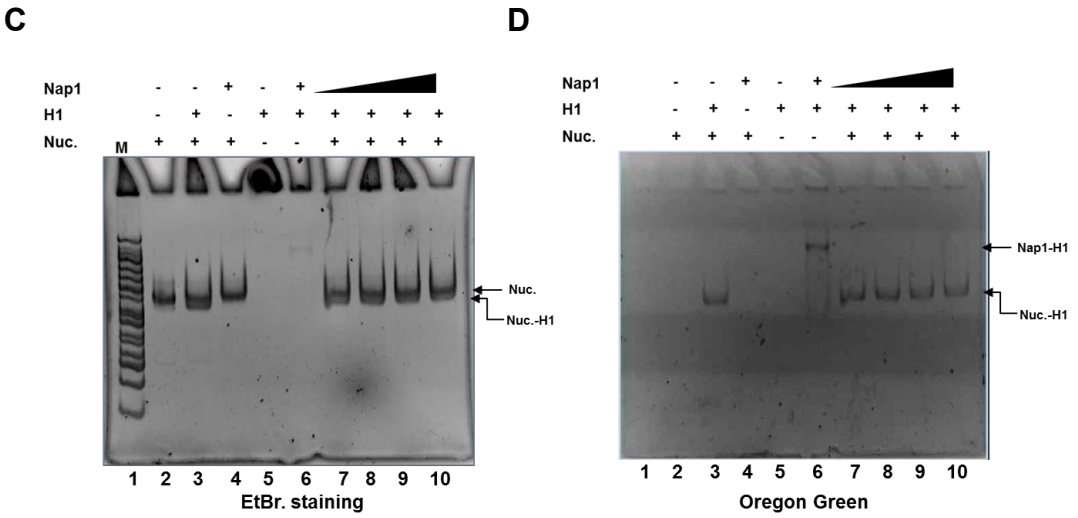


Figure I.3. Neither FACT or Nap1 removes H1 from nucleosome. To determine if FACT or Nap1 removes H1 from nucleosome, 100 nM 207 nucleosome was mixed with 50 nM H1 first, and then FACT or Nap1 (50 nM to 1.6 μM) was added into the reaction mixture. Nucleosome-H1 complex was analyzed on a 5% PAGE, and visualized by EtBr (A and C) and fluorescence (B and D). staining and fluorescence (H1). It was observed that H1 is not removed as FACT or Nap1 is titrated. Similarly, no FACT or Nap1-H1 complexes were observed either (B and D lane7-10).

nucleosome (Stutzer et al. 2016). In the future, we intend to test if histone chaperones work cooperatively to displace H1 from chromatin. Also, we plan to test if histone modifications occurring at core histones facilitate histone chaperone function in regulation of H1 dynamics.

I.6 Acknowledgement

We would like to thank Alison E. White for generously providing labeled mouse linker H1 histone. We are also grateful for the valuable suggestions from Dr. Sheena D'Arcy and Dr. Duane D. Winkler.