Solid-state NMR studies of chromatin structure and dynamics

Prasanna, Chinmayi

2020

Prasanna, C. (2020). Solid-state NMR studies of chromatin structure and dynamics. Doctoral thesis, Nanyang Technological University, Singapore.

<https://hdl.handle.net/10356/146519>

<https://doi.org/10.32657/10356/146519>

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**NANYANG
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SINGAPORE

**SOLID-STATE NMR STUDIES OF CHROMATIN
STRUCTURE AND DYNAMICS**

CHINMAYI PRASANNA

SCHOOL OF BIOLOGICAL SCIENCES

2020

Solid-State NMR Studies of Chromatin Structure and Dynamics

Chinmayi Prasanna

SCHOOL OF BIOLOGICAL SCIENCES

A thesis submitted to the Nanyang Technological
University in partial fulfilment of the requirement for the
degree of Doctor of Philosophy

2020

Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research done by me except where otherwise stated in this thesis. The thesis work has not been submitted for a degree or professional qualification to any other university or institution. I declare that this thesis is written by myself and is free of plagiarism and sufficient grammatical clarity to be examined. I confirm that the investigations were conducted in accord with the ethics policies and integrity standards of Nanyang Technological University and that the research data are presented honestly and without prejudice.

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Prof. Lars Nordenskiöld

Authorship Attribution Statement

This thesis contains material from two papers published in the following peer-reviewed journals in which I am listed as an author.

- Chapter 3 and sections 5.3.1, 5.3.2 from Chapter 5 are published as Shi, X. Y., C. Prasanna, T. Nagashima, T. Yamazaki, K. Pervushin, and L. Nordenskiöld. Structure and Dynamics in the Nucleosome Revealed by Solid-State NMR. *Angewandte Chemie-International Edition* **57**(31), 9734-9738 (2018). DOI: 10.1007/s12104-020-09927-w

The contributions of the co-authors are as follows:

- Lars Nordenskiöld and Konstantin Pervushin co-designed the project.
 - I prepared the samples and analyzed the sample quality. With assistance from Xiangyan Shi, samples were packed in SSNMR rotors.
 - Xiangyan Shi carried out the SSNMR spectroscopic measurements, processed and analyzed the data.
 - Xiangyan Shi prepared the manuscript drafts. I contributed to the sample preparation section in the supplementary information. The manuscript was revised by Lars Nordenskiöld and Konstantin Pervushin.
 - Preliminary investigations of this study were carried out by our collaborators-T. Nagashima, T. Yamazaki at RIKEN, Japan.
- Sections 4.2, 4.3.1, and 4.3.2 from Chapter 4 are published as Shi, X. Y., C. Prasanna, K. Pervushin, and L. Nordenskiöld. Solid-state NMR ^{13}C , ^{15}N assignments of human histone H3 in the nucleosome core particle. *Biomolecular NMR Assignments*. **14**(1), 99-104 (2020). DOI: 10.1002/anie.201804707

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- Xiangyan Shi and I carried out the SSNMR spectroscopic measurements. Xiangyan Shi processed and analyzed the data.
- I together with Xiangyan Shi prepared the manuscript drafts. The manuscript was revised by Lars Nordenskiöld and Konstantin Pervushin.
- Sections 4.3.3, 4.3.4, and 4.4 from Chapter 4 and section 5.4 from Chapter 5 are published as Shi. X. Y., C. Prasanna, A. Soman, K. Pervushin, and L. Nordenskiöld. Dynamic networks observed in the nucleosome core particles couple the histone globular domains with DNA. *Communications Biology*. **3**, 639 (2020). DOI: 10.1038/s42003-020-01369-3

The contributions of the co-authors are as follows:

- Lars Nordenskiöld and Konstantin Pervushin co-designed the project.
- I prepared the histones, histone octamer, analyzed the sample quality, and packed them in SSNMR rotors.
- Aghil Soman prepared the telomeric DNA and reconstituted the telomeric NCP.
- Xiangyan Shi and I carried out the SSNMR spectroscopic measurements. Xiangyan Shi processed and analyzed the data.
- Xiangyan Shi prepared the manuscript drafts and I and Aghil contributed to the sample preparation sections. The manuscript was revised by Lars Nordenskiöld and Konstantin Pervushin.

21st August 2020

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Date

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Chinmayi Prasanna

Acknowledgments

Utmost gratitude to my supervisor Prof. Lars Nordenskiöld for offering me an opportunity in his group here at the school of biological science (SBS), to work in the area of chromatin biology, using solid-state NMR spectroscopy. *“Lars, you are a fantastic supervisor any Ph.D. student can ever have, I am extremely fortunate. You have always encouraged students in the lab to independently carry out experiments fostering critical scientific thinking. I appreciate the time you give us as a PI despite your administrative commitments in academia. I have learned a great deal of multi-tasking from you. I am indebted to your invaluable guidance in conducting this research work for the Ph.D. degree. Thank you very much”*

I would like to thank my co-supervisor Assoc. Prof. Konstantin Pervushin (developed the TROSY experiment). *“Thank you, Konstantin, for the NMR spectroscopy course, you taught, which greatly enhanced my fundamental knowledge. As a teaching assistant for your courses, I acquired skills to explain the complex concepts in structural biology and population genetics, in simple terminology to undergrads. Thanks for your critical inputs on this project, my presentation throughout the four years”*

The most important person without whom this work would be impossible is Dr. Xiangyan Shi. *“Xiangyan, I couldn’t have had a better mentor than you to learn biological SSNMR spectroscopy. Eternally grateful to you for the training and for your patience in teaching SSNMR to a person with a molecular biology background. To me, you are a motivating; profoundly knowledgeable person and a treasure in the field of biological SSNMR. You have been the driving force of this fantastic project. I could understand the basic concepts in biological SSNMR, thanks to you. I have learned effective research writing and presentation from you. You are the best colleague and now a good friend. Heartfelt thanks!”*

I would like to thank Aghil, Qinming, Sai, and Jaclyn for their assistance in getting acclimatized to the lab in my initial days. *“Aghil and Qinming, thanks for making me familiar with chromatin protocols, timely wet lab tips that made a huge difference in reducing experimental time”*. *“Jaclyn, I made unforgettable memories with you in lab and conference”* I am lucky to have a best friend in my colleague Vishal who was always there for me. *“Vishal, I thank you for your assistance in getting familiar with NTU in my early days, your assistance in compiling tools on Linux, and, for accompanying me to numerous coffee/lunch/beer sessions,*

during/after work”. I thank N.Korolev- *“Thank you big Niki, for your valuable feedback in putting together this thesis, your dedication to research motivates me to perform better”*, I thank the rest of my colleagues–N. Berezhnoy, Sasha, Terry, Bhuvana for creating a conducive work environment. *“I tremendously benefitted through scientific discussions with all of you during the journal club meetings; cultural and worldly discussions during happy hours and Lars lab Christmas parties”* I also thank Artur, Tiago, and Lifang from the CyanoSynBio@NTU lab with who we shared our wet lab space.

I would like to thank my thesis advisory committee members- Assoc. Prof. Jaume Torres (SBS) and Prof. Phan Anh Tuan (SPMS) for their critical comments, invaluable suggestions, feedback, and intriguing questions.

I acknowledge the usage of NTU Center of High Field NMR Spectroscopy and Imaging. I thank my school- SBS for being student-friendly and providing a great scientific environment for research; happy hours fostering networking sessions not only with fellow students but with various PIs. I thank NTU for the research scholarship. I acknowledge the funds from Tier 2 (MOE2018-T2-1-112) and Tier 3 Grants (MOE2012-T3-1-001) by the Singapore Ministry of Education (MOE) to carry out this research work. I thank all the SBS office staff, especially Fransisca, May, Victor, and Raymond for supporting me throughout these years.

I am obliged to my first research supervisor- Prof. Hanudatta Shastry Atreya for motivating and pushing me to pursue a Ph.D. in the field of biological NMR *“Gone too soon, you would have been the happiest person to read through this work if you were alive. Thank you for your constant support throughout my research career”*. I thank Prof. Anil Kumar (developed the NOESY experiment), Prof. K. V. Ramanathan, Prof. N. Surya Prakash, and Dr. Ragothama from NRC, IISc, Bangalore for the NMR courses they taught and the constant support they have been offering to date. *“NMR may be a tool, but it became a stepping-stone in my research career because of all of you. You guys pushed me even when I doubt myself. Thanks for bolstering me and everyone from NRC in different parts of the world.”*

“Thank you, guys–Dzeneta, Antareep, Spandan, Shuddhodana, Stephanie, Harvey, Jurek, Viktor, Christina, Vivek, Divya, and Gitanjali for making Ph.D. a memorable journey.”

Last but not the least, I am grateful to my father-Prasanna Shiva Kumar for nourishing my dream and encouraging my passion to study science in an engineering crazy nation. I thank my mother-Saroja and my brother-Shashidhar for their constant love and support.

Dedicated to
Late. Prof. Hanudatta Shastry Atreya
(1974-2020)

Abstract

Understanding the dynamic organization of chromatin is indispensable as it provides crucial information to delineate the cellular processes that it mediates – DNA replication, transcription, and repair. The nucleosome core particle (NCP) is the fundamental unit of eukaryotic chromatin composed of four core histone proteins (H2A, H2B, H3, and H4) organizing ~147 bp of DNA. While the structure of the nucleosome is understood predominantly using X-ray crystallography and cryo-EM, quantitative information about nucleosome dynamics remains elusive. Our objective is to address this gap in understanding the chromatin structure and dynamics using solid-state nuclear magnetic resonance (SSNMR) spectroscopy. SSNMR spectroscopy is a state-of-the-art technology to probe protein dynamics at the atomic-resolution.

To begin with, we have conducted dipolar-coupling and J-coupling based SSNMR experiments for the globular domain and the tail regions of human histones in the NCP. We obtained unambiguous chemical shift assignments and secondary structure. In the next step, we carried out experiments on histones hH4 and hH3 in the NCP to probe site-specific motions in the nanosecond to microsecond and microsecond to millisecond timescales. Furthermore, we investigated the influence of histone dynamics in the context of nucleosomes in chromatin fibers (nucleosome arrays) and, the DNA sequence variation using human telomeric repeat.

The results indicate that the well-structured globular domains of histones in the nucleosome array and telomeric NCP have the same conformation as in the 601-NCP. While the histones in the nucleosome array exhibit minor conformational changes in few regions, heightened dynamics were observed across the histones hH4 and hH3 in the telomeric NCP, relative to 601-NCP. Histone N-terminal tail domains adopt similar structures in the NCPs and the nucleosome array, indicating that the tails are available for interactions regardless of the level

of chromatin compaction. Histone tails in the telomeric NCP are relatively flexible, likely due to the enhanced flexibility of the DNA in the telomeric NCP.

Overall, we have demonstrated the feasibility and applicability of SSNMR spectroscopy in understanding the structure and dynamics of histones in the nucleosome at atomic resolution and physiological chromatin concentrations. Ours is the first SSNMR based study to have probed motions and their amplitudes in histones in the chromatin. Moving forward, we aim to carry out these studies on other histones and DNA. We are currently working on SSNMR based heterochromatin binding protein (HP1) mediated chromatin compaction. Overall, our study sheds light on histone quantitative dynamics with potential contributions to understand the effects on the chromatin-mediated dynamic processes—nucleosome stability, sliding, unwrapping, and DNA accessibility.

Abbreviations

12-mer array	nucleosome array containing 12 repeats of 177 bp telomeric DNA
601-NCP	nucleosome core particle formed by 145 bp 601 'Widom' DNA
APS	ammonium persulfate
bp	base pair
CIA	chloroform-isoamyl alcohol
CP	cross-polarization
CP MAS	cross-polarization magic angle spinning
DARR	dipolar assisted rotational resonance
DMSO	dimethyl sulfoxide
DNA	deoxyribose nucleic acid
DTT	1, 4-dithio-d-threitol
<i>E. coli</i>	<i>Escherichia coli</i>
EDTA	ethylene diamine tetraacetic acid
FPLC	Fast purification liquid chromatography
hH3	human histone H3
hH4	human histone H4
hHO	human histone octamer
HO	histone octamer
IPTG	isopropyl β -d-1-thiogalactopyranoside
MAS	magic angle spinning
MWCO	molecular weight cut-off
NCP	nucleosome core particle
NMR	nuclear magnetic resonance
NRL	nucleosome repeat length
OD	optical density
PTM	post-translational modification
RB	reconstitution buffer
SSNMR	solid-state nuclear magnetic resonance
Telo NCP	nucleosome core particle formed by 145 bp telomeric DNA
TEMED	tetramethyl ethylene diamine

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Introduction

1. INTRODUCTION

We have traversed a long way in the journey of understanding the organization of the genetic material, DNA, from the double helix structure (Watson and Crick 1953) to its organization into chromatin (Kornberg 1974) and the fundamental unit – the nucleosome core particle (NCP) (Luger, Mader et al. 1997, Davey, Sargent et al. 2002). Organizing more than two-meters long human DNA in the nucleus of every single cell into a compact structure called chromatin (Figure 1) is a crucial task in the cell nucleus for genome protection. The genome has to be decoded periodically to carry out basic functions such as DNA replication, transcription and DNA repair and transcription to achieve cell growth and development, which are central to the continuity of life.

DNA is a negatively charged long biopolymer. In eukaryotes, DNA is compactly packed inside the nucleus of the cell by proteins called histones, which comprise many positively charged amino acids rendering them a large net positive charge. There are four core histone proteins, H2A, H2B, H3, and H4 that form histone octamer (HO). Roughly 147 bp DNA is wrapped around each HO to form a left-handed ~1.65 turn superhelix, constituting the basic unit of chromatin, the nucleosome core particle (NCP). The linker histone further packs NCPs by binding to the linker DNA that spans between the NCP (Hergeth and Schneider 2015). This short-range internucleosomal interaction is central to the compaction of DNA into higher-order structures, the chromatin fiber. A unit of NCP with linker histones forms a “chromatosome” organizing about 167 bp of DNA.

Various non-histone binding proteins perform diverse functions. One type of those proteins modulates the long-range chromatin interactions and condensation. Heterochromatin protein 1 (HP1) plays an important role in the formation of heterochromatin (Kumar and Kono 2020). DNA in the heterochromatin regions are more densely packed and less accessible in

comparison with those in the euchromatic regions. This hierarchical organization (Figure 1) serves the function of genome protection regulating the access of cellular proteins that perform the crucial tasks of DNA replication, transcription activation or repression, DNA repair, etc. (Grant 2001). The interplay between transcription factors and the nucleosomes results in DNA translocation and changes of transcription state (Teichmann, Wigge et al. 2012).

Enzymes that covalently modify histones facilitate and/or restrict the accessibility of the nucleosomal DNA to chromatin remodelers. For example, enzymes that bring about post-translational modification (PTM)—acetylation, methylation, phosphorylation, ubiquitination, etc. among many others, are crucial for chromatin-protein interactions, and in recruiting enzymes that bring about chromatin remodeling. Chromatin remodelers are ATP-dependent remodelers like SWI/SNF, ISWI, INO80, and NuRD/Mi-2/CHD, which move along the nucleosomes expediting various chromatin transformations (Varga-Weisz 2001). High Mobility Group (HMG) architectural proteins compete with linker histone for linker DNA binding, thereby promoting the decondensation of chromatin (Bustin 2001). Histone chaperones facilitate incorporation/removal of either/or canonical and variant histones performing the crucial task of assembly and disassembly of the nucleosome in distinct phases of the cell cycle (Hammond, Stromme et al. 2017).

Chromatin is a dynamic entity as it is a hub for chromatin templated processes. Understanding the structure and organization of chromatin and its fundamental unit in the dynamic context of the cell is indispensable as it has fundamental ramifications in our understanding of cellular processes like DNA replication, transcription, and DNA repair. Chromatin plays a vital role in their regulation in two ways- either promotion or disruption which can lead to numerous manifestations in a cell eventuating in diseases, cancer, aging, etc (Zane, Sharma et al. 2014, Evans, Horrell et al. 2019).

Figure 1. The higher-order organization of DNA into chromatin. Reprinted from (Fyodorov, Zhou et al. 2017). Copyright Springer Nature

1.1. ORGANIZATION OF THE NUCLEOSOME

Figure 2. Organization of the Nucleosome Core Particle. Organization of the nucleosome. (A) The overall structure of the nucleosome core particle at 1.9 Å resolution PDB ID: 1KX5 (Davey, Sargent et al. 2002). (B) Domain organization of the four core histones. The α -helices are depicted as boxes (labeled as $\alpha1$, $\alpha2$, and $\alpha3$), while the intervening loops are shown as lines (labeled as L1 and L2). The

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The fundamental organization of NCP structure was revealed in 1997 by X-ray crystallography (Luger, Mader et al. 1997) followed by the determination of higher resolution structures later (Davey, Sargent et al. 2002). The octameric core complex is made of four histones- H2A, H2B, H3, and H4. Individual histones are characterized by the well-structured globular domain encompassed by three helices ($\alpha 1$, $\alpha 2$, and $\alpha 3$) which are connected by the loop regions (L1 and L2) (Luger, Mader et al. 1997). Furthermore, the globular domain of H3 has a short αN helix connected to the $\alpha 1$ helix by the LN loop. The globular domains of histones are flanked by the highly mobile tail regions on their N and C termini. While H3 has the longest N-terminal tail, H2A has the shortest (Figure 2B). H2A forms a heterodimer with H2B and H3 with H4 forming a histone-fold heterodimer handshake motif (Figure 2C). Two heterodimers of H2A-H2B binds with opposite halves of the H3-H4 tetramer forming the histone octamer (HO) complex (Figure 2D).

HO functions like a bobbin wrapping the DNA around it. Arginine from histones makes contact with the negatively charged phosphate groups on the DNA superhelix at regular intervals (Figure 2E). β bridges and paired $\alpha 1$ region from heterodimers H2A-H2B and H3-H4

make contacts with the minor groove of the DNA (Figure 2C). For example, there are about seven such histone-DNA contact sites in the X-ray structure of NCP PDB ID: 1KX5 (Davey, Sargent et al. 2002)) (Figure 2E). The flexible N-terminal tails extend from the nucleosome core significantly contributing to histone mediated interactions. There are eight N-terminal tails that extend from the eight histones and two C-terminal tails from histone H2A. The amino acids E56, E61, E64, D90, E91, E92 of H2A, and E102, E110 of H2B on the surface of the core constitute the so-called ‘acidic patch’ (Luger, Mader et al. 1997) (Figure 2F).

Both the globular domains and the tail regions of histones are subjected to PTMs as discussed above. Histone core PTMs regulate nucleosome structure and dynamics, thus central to chromatin compaction, DNA replication, transcription, and repair (Tessarz and Kouzarides 2014). Histone tail PTMs are pivotal for chromatin compaction and DNA repair. Some PTMs on the lateral surfaces of histones (on both globular and tail domains) are essential for gene expression (Lawrence, Daujat et al. 2016). Known histone PTMs on both histone globular domain and tail regions are listed in Table 1 and Table 2 respectively. These modifications on both globular and tail regions play a pivotal role not only in chromatin structure and dynamics but also in gene regulation (Lawrence, Daujat et al. 2016). While modifications on histone N-terminal tail and their binding mechanisms have been extensively studied, similar studies on histone globular domain that also regulate structure and dynamics are limited (Mersfelder and Parthun 2006, Tessarz and Kouzarides 2014).

Histone globular domain modifications*			
Histone	Site	Modification	Role
H2A	H2AK36	Acetylation	
	H2AK74	Methylation	
	H2AK75	Methylation	DNA binding
	H2AK99	Methylation	
	H2AQ105	Methylation	rDNA transcription
	H2AK119	Acetylation	
	H2AK119	Ubiquitylation	
H2B	H2BK31	Methylation	
	H2BS33	Phosphorylation	DNA binding, cap of the double helix
	H2BK40	Methylation	Indirect DNA binding
	H2BR76	Methylation	Charge neutralizes negative helix dipole
	H2BK82	Acetylation	
	H2BR83	Methylation	DNA binding
	H2BR89	Methylation	
	H2BR96	Methylation	
	H2BK105	Acetylation	
	H2BK113	Acetylation	
	H2BK117	Acetylation	
H3	H3Y41	Phosphorylation	Transcription
	H3R42	Methylation	Transcription
	H3T45	Phosphorylation	Apoptosis
	H3R52	Methylation	Indirect (H ₂ O-mediated) DNA binding
	H3R53	Methylation	
	H3K56	Acetylation	Indirect DNA binding, transcription, replication, repair
	H3K56	Methylation	Transcription, replication, repair
	H3K64	Acetylation	Transcription
	H3K64	Methylation	Transcription
	H3K79	Methylation	Transcription
	H3K115	Acetylation	Indirect DNA binding
	H3T118	Phosphorylation	DNA binding, H4 Arg46 binding Transcription, repair
	H3K122	Acetylation, methylation, formylation	Transcription, oxidative damage (formylation)
H4	H4K31	Acetylation	Indirect DNA binding
	H4S47	Phosphorylation	Indirect DNA binding
	H4K59	Methylation	Salt bridge with H4 Glu63
		formylation	Oxidative damage (formylation)
	H4K77	Acetylation	Indirect DNA binding
	H4K79	Acetylation, formylation	Oxidative damage (formylation)
	H4K91	Acetylation ubiquitylation, succinylation, butyrylation, citrullination, propionylation	Forms salt bridge with H2B Glu63 Replication, DNA damage
	H4R92	Methylation	

Table 1. Histone globular domain modifications. Table adapted from (Cosgrove, Boeke et al. 2004, Tessarz and Kouzarides 2014, Lawrence, Daujat et al. 2016). The role of some modifications that are not known, is left blank. *This is by no means an exhaustive list.

Histone tail modifications*		
Histone	Modification	Role
H2A	H2AS1P	Mitosis; chromatin assembly
	H2AK4/5ac	Transcriptional activation
	H2AK7ac	Transcriptional activation
	H2AK119P	Spermatogenesis
	H2AK119uq	Transcriptional repression
H2B	H2BS14P	Apoptosis
	H2BS33P	Transcriptional activation
	H2BK5ac	Transcriptional activation
	H2BK11/12ac	Transcriptional activation
	H2BK15/16ac	Transcriptional activation
	H2BK20ac	Transcriptional activation
	H2BK120uq	Spermatogenesis/meiosis
	H2BK123uq	Transcriptional activation
H3	H3K4me2	Permissive euchromatin
	H3K4me3	Transcriptional elongation; active euchromatin
	H3K9me3	Transcriptional repression; imprinting; DNA methylation
	H3R17me	Transcriptional activation
	H3K27me3	Transcriptional silencing; X-inactivation; bivalent genes/gene poising
	H3K36me3	Transcriptional elongation
	H3K4ac	Transcriptional activation
	H3K9ac	Histone deposition; transcriptional activation
	H3K14ac	Transcriptional activation; DNA repair
	H1K18ac	Transcriptional activation; DNA repair; DNA replication
	H3K23ac	Transcriptional activation; DNA repair
	H3K27ac	Transcriptional activation
	H3T3P	Mitosis
	H3S10P	Mitosis; meiosis; transcriptional activation
	H3T11/S28P	Mitosis
H4	H4R3me	Transcriptional activation
	H4K20me1	Transcriptional silencing
	H4K20me3	Heterochromatin
	H4K5ac	Histone deposition; transcriptional activation; DNA repair
	H4K8ac	Transcriptional activation; DNA repair; transcriptional elongation
	H4K12ac	Histone deposition; telomeric silencing; transcriptional activation; DNA repair
	H4K16ac	Transcriptional activation; DNA repair
	H4S1P	Mitosis

Table 2. Histone tail modifications. Table adapted from (Lawrence, Daujat et al. 2016). *This is by no means an exhaustive list.

There are over 200 structures of nucleosome cores deposited in the Protein Data Bank (PDB) (<https://www.rcsb.org/pdb>), determined by X-ray diffraction (XRD), cryo-electron microscopy (cryo-EM), and nuclear magnetic resonance (NMR) spectroscopy. Molecular structure and physicochemical features of NCP, and the nucleosome-nucleosome stacking (in more than 140 published structures) have been systematically studied (Korolev, Lyubartsev et al. 2018). This a numerical description of NCP stacking in chromatin systems with the usage

of an “NCP-centered” co-ordinate system. The variation in crystal structures in the PDB can also be attributed to the usage of different DNA sequences (both natural and artificial), DNA of variable length, canonical and variant histones, and nucleosome with interacting proteins. Undoubtedly, X-ray crystallography played a compelling role in understanding the fundamental organization of the NCP (Richmond, Finch et al. 1984, Luger, Mader et al. 1997, Davey, Sargent et al. 2002, McGinty and Tan 2015). Whilst, NMR spectroscopy has not only been conducive in establishing the flexible nature of the N-terminal tail regions (Lilley, Howarth et al. 1976), but also in understanding the structure of linker histone H1 and H5 (Clore, Gronenborn et al. 1987) and the chromatin remodeler HMGN2 in complex with nucleosome (Kato, van Ingen et al. 2011). It is challenging to crystallize large protein complexes and obtain high-resolution structures with details at the atomic level. With the advent of cryo-electron microscopy and the resolution revolution, it has been possible to extend the chromatin studies to examine the histone modifications and structural flexibility in large chromatin complexes (Wilson and Costa 2017), and probe the higher-order in the chromatin fiber (Song, Chen et al. 2014).

1.2. NMR SPECTROSCOPY FOR STRUCTURAL STUDIES OF CHROMATIN

Dynamic regulation of chromatin structure is paramount in manifestations of almost all the biological events in a cell—dynamics of gene regulation during early mouse embryo development (Koch 2020), learning and memory in the central nervous system (Sweatt 2016), interconversion of interphase chromatin to a mitotic chromosome (Kinoshita and Hirano 2017). Changes in individual nucleosome structure and dynamics have been shown to control the higher-order structure of chromatin (Montel, Menoni et al. 2009). A study on histone dynamics in the context of herpes simplex virus (HSV-1) genes integrating into the human genome

provided crucial information about the accessibility of the viral DNA to transcription factors during the lytic stages of infection (Hu, Depledge et al. 2019).

NMR spectroscopy is one of the complementary techniques apart from small-angle X-ray scattering (SAXS), fluorescence resonance energy transfer (FRET), hydrogen/deuterium exchange coupled with mass spectrometry (HDX-MS) and electron microscopy (EM) which can aid in filling the gaps in the structural characterization of the NCP using X-ray crystallography, particularly to apprehend chromatin dynamics at atomic resolution. (Fierz and Poirier 2019, Zhou, Gaullier et al. 2019).

Histone N-terminal tails are largely unresolved in the electron density map even in the high-resolution X-ray structures, To understand their contribution to chromatin architecture and DNA accessibility by various chromatin factors, structural techniques that aid in resolving these flexible regions are a requisite. NMR is well known to be used in such studies of flexible regions and intrinsically disordered proteins (IDPs) (Kosol, Contreras-Martos et al. 2013). Besides, NMR has the potential to reveal detailed atomic information on the structure and dynamics of nucleosome arrays and reconstituted chromatin fiber systems and shall show higher tolerance to the variable conditions such as salt concentration and DNA templates compared to X-ray crystallography or cryo-EM methods.

One of the earliest NMR investigations resulted in an understanding that the amino acids alanine, leucine, isoleucine, and valine from the helical regions of histones interacted with the nucleosomal DNA. Using one-dimensional ^1H , ^{13}C , and ^{31}P NMR experiments, the conformation of the nucleosomal DNA was inferred (Bradbury, Crane-Robinson et al. 1967). (Kallenbach, Appleby et al. 1978, Feigon and Kearns 1979). Using NMR in consortium with CS-Rosetta (chemical-shifts based protein structure prediction tool), the solution structure of

the H2A-H2B dimer has been determined providing insights about the hetero-dimer dynamics contribution in chromatin (Moriwaki, Yamane et al. 2016).

1.2.1. Histone tails

NMR has significantly contributed to our understanding of histone N-terminal tail structure and dynamics. Using hydrogen exchange and NMR, histone H3 N-terminal tails were found to be highly dynamic in the NCP, whereas they form stable structures in the nucleosome array (Kato, Gruschus et al. 2009). Contrarily, using SSNMR it was demonstrated that the N-terminal tails of histones H3 and H4 are highly dynamic even at a high level of compaction (Gao, Nadaud et al. 2013). Using solution NMR, only the residues 1-15 in histone H4 in the NCP were detected, indicating that the residues 16-22 in tails of both histone H4 fold onto the neighboring nucleosome. Furthermore, mutational disruption (acetylation mimic) of these residues is reported to cause structural disorder in the N-terminal basic patch which inhibits chromatin compaction (Zhou, Feng et al. 2012). Solution NMR studies of histone tails have revealed the mechanistic insights on PTMs and linker histone binding reduce the tail dynamics of histone H3 (Stutzer, Liokatis et al. 2016).

1.2.2. Linker histones

The role of solution NMR in structural studies of linker histones in chromatosome and NCP has been significant from the earliest days of structural studies of chromatin (Bradbury, Carpenter et al. 1973). An array of NMR studies contributed in understanding the amino acid composition-lysine rich (positively charged); secondary structure; binding mode of linker histone- H1 to the nucleosomal DNA; its role in chromatin condensation (Bradbury, Cary et al. 1975, Bradbury, Danby et al. 1975, Chapman, Hartman et al. 1976, Hartman, Chapman et al.

1977, Aviles, Chapman et al. 1978, Aviles, Danby et al. 1979, Tancredi and Temussi 1979). In parallel, the first partial chemical shift assignment of avian linker histone-H5 (Chapman, Aviles et al. 1978) was reported followed by a complete assignment and secondary structure determination (Zarbock, Clore et al. 1986). With technical advancements in NMR spectroscopy and isotope labeling, after more than a decade, complete chemical shift assignments were obtained and the secondary structure of the globular domain of histone H1 was derived (Cerf, Lippens et al. 1993) and compared with the globular domain of histone H5 to highlight the molecular basis of the different affinities in their DNA binding (Cerf, Lippens et al. 1994).

An NMR structural model of the globular domain of H1 in complex with the nucleosome has suggested different binding geometries (Zhou, Feng et al. 2013). Different binding modes (on-dyad or off-dyad) of linker histones; amino acids involved in the interaction and their corresponding implications in the formation of higher-order structures have been thoroughly investigated for the H5 globular domain (Zhou, Jiang et al. 2015).

1.2.3. Methyl-TROSY based NMR studies

Owing to the high molecular weight of the NCP, many structural biologists studying chromatin have used the combination of methyl-labeling and methyl-TROSY (Transverse Relaxation Optimized Spectroscopy) (Pervushin, Riek et al. 1997, Wiesner and Sprangers 2015) to study chromatin and interacting complexes in solution. In a first of its kind decisive study, using methyl- TROSY, the nucleosome binding sites of HMGN2 (high mobility group nucleosomal protein) have been identified, revealing that it interacts with both the acidic patch and the nucleosomal DNA near the entry/ exit point (Kato, van Ingen et al. 2011). Interactions that drive the chromosome segregation in mitotic events have been revealed to determine the mechanism of CENP-C (centromeric protein-C) recognizing CENP-A (histone variant of

centromere), using the combination of methyl-based chemical shift perturbation and paramagnetic relaxation enhancement NMR (Kato, Jiang et al. 2013).

Combining methyl-TROSY based NMR with other techniques including the MD simulations, the binding of RNF 168 (RING domain) to the acidic patch on nucleosome (Horn, Uckelmann et al. 2019) and ability of RNF169 (UDM2 domain) (ubiquitin E3 ligases- involved in DNA double-strand break repair) to distinguish numerous ubiquitination states of chromatin was revealed (Kitevski-LeBlanc, Fradet-Turcotte et al. 2017). Binding sites were mapped both on RNF 168 and RNF169 and its interacting partner, ubiquitylated H2A, in the nucleosome. Another example of methyl-TROSY based NMR in consolidation with other techniques showed that the histone octameric core undergoes distortion upon mobilization by an ATP dependent remodeler, SNF2H (Sinha, Gross et al. 2017). The inherent plasticity of nucleosomes plays a crucial role in the structure and dynamics of chromatin (Kitevski-LeBlanc, Yuwen et al. 2018).

1.2.4. Others

Complete structure determination of few histone chaperones and their binding sites on the histones have been identified (Mousson, Lautrette et al. 2005, Su, Hu et al. 2011). NMR has also been used to monitor the real-time enzyme activities of histone deacetylases (HDACs) and histone acetyl-transferases (HATs) (Dose, Liokatis et al. 2011). Time-resolved NMR spectroscopy has been used to study the asymmetric histone modification crosstalk, which is otherwise arduous to understand (Liokatis, Klingberg et al. 2016).

Solution NMR based studies are not limited to histones as some researchers applied it to the nucleosomal DNA. The role of pre-existing minor groove conformations in the formation of nucleosome has been probed using ^{31}P NMR spectroscopy (Xu, Ben Imeddourene et al.

2014). Methyl-TROSY based NMR studies of chromatin are well known as discussed in the previous sections. Using a rather innovative approach of simultaneous enzymatic methylation of several sites on DNA, Abramov et.al demonstrated the usage of methyl-TROSY NMR experiment to deduce the effects of multiple methylations of nucleosomal DNA on the histone core (Abramov, Velyvis et al. 2020).

The contribution of solution NMR has been astounding in the field of chromatin biology, notably in understanding the dynamic nature of histone tails and the structure of linker histones. Nonetheless, histone core domain studies have been restricted to methyl-TROSY based approaches, usage of paramagnetic tags, H/D exchange studies on a few sites, or stretches of peptides. This is partly attributed to the molecular weight limitation, imposing technical challenges in the structural characterization of the NCP bound to chromatin factors. Additionally, histones and their PTMs in solution NMR are mainly probed based on backbone amide detection. Since cellular chromatin concentrations are very high (Bohrmann, Haider et al. 1993, Bertin, Mangenot et al. 2007), probing the structure and dynamic properties in these conditions *in vitro* are important to understand the biological implications. Studying NCPs using solution NMR is also limited by severe line broadening due to the slow molecular tumbling. Studying longer arrays of chromatin in a cell-like environment at atomic resolution using solution NMR is a seemingly difficult task. A potential technique that can address these challenges is solid-state NMR spectroscopy.

1.3. SSNMR SPECTROSCOPY FOR THE STUDY OF CHROMATIN STRUCTURE AND DYNAMICS

SSNMR does not suffer from the molecular weight limitations and internal motions are readily accessible because of the absence of the molecular tumbling. SSNMR spectroscopy is a state-of-the-art method to study intermolecular interactions and protein dynamics. With a

giant leap of technological innovation in NMR hardware and methodology development in recent years (Schanda and Ernst 2016), the usefulness of SSNMR spectroscopy to study the structure and dynamics of various biological macromolecules has become evident (Mandala, Williams et al. 2018, van der Wel 2018). It is the versatility of the technique that has been used to solve complete three-dimensional structures of large proteins, membrane proteins, protein-protein, protein-RNA, and protein-DNA complexes which are not amenable to solution NMR studies (Renault, Cukkemane et al. 2010). A range of biological samples of variable nature can be studied using SSNMR giving us atomic insights from both microscopic molecular structure of protein micro-crystals (Knight, Pell et al. 2012) to macroscopic molecular packing in sedimented proteins, fibrils for example amyloid fibrils (Comellas and Rienstra 2013), gels, etc.

The very first application of SSNMR spectroscopy in chromatin biology was in 2013, revealing the histone N-terminal tails were highly dynamic irrespective of their level of compaction in the nucleosome arrays. Besides, dynamic residues in the N-terminal tails of H3 and H4 in the nucleosome arrays were identified at physiological conditions (Gao, Nadaud et al. 2013). While, a previous study reported that the N-terminal tails of histones were stabilized in arrays and mobile in the NCPs (Kato, Gruschus et al. 2009). This sheds light on the importance of the choice of methodology and experimental conditions in discerning a biological problem through *in vitro* studies. In a recent highly interesting work, the structure and dynamics of the globular and tail domains of histone H2A in sedimented nucleosomes were divulged in a site-specific manner using SSNMR (Xiang, le Paige et al. 2018). The authors also mapped the binding sites of LANA protein (that binds to the acidic patch) on the nucleosome surface using the backbone amide resonances obtained. An important revelation was made, that LANA is bound to both copies of the histone H2A in the nucleosome and not

just one single copy as previously determined by X-ray crystallography (Barbera, Chodaparambil et al. 2006).

1.4. BASIC CONCEPTS IN SSNMR SPECTROSCOPY

In SSNMR, the interactions such as chemical shift anisotropy (CSA) and dipolar coupling largely contribute to line broadening. Hence, line narrowing techniques become pivotal in this direction, though it requires meticulous analysis. Techniques such as magic angle spinning (MAS) and proton decoupling are commonly utilized to obtain high-resolution SSNMR spectra. In addition, cross-polarization (CP) is often employed for sensitivity enhancement and heteronuclear magnetization transfer.

In SSNMR, the nuclei interactions are orientation-dependent, resulting in broad peaks called “powder pattern”. While in its solution counterpart, many anisotropic interactions are averaged out by the rapid Brownian tumbling. In solids, where molecular tumbling is absent, MAS (spinning the sample at an angle of 54.74° , called magic angle with respect to the axis of B_0 field) is utilized to reduce the anisotropic nuclear spin interactions (Andrew, Bradbury et al. 1959, Andrew, Bradbury et al. 1960). MAS can be combined with other methods discussed below to obtain high-resolution spectra.

Figure 3. Magic angle spinning (MAS) in an external magnetic field

1.4.1. Cross-Polarization (CP)

Dilute spin $\frac{1}{2}$ nuclei- ^{15}N , ^{13}C have longer relaxation times in solids, resulting in longer relaxation delays increasing the experimental time. Transfer of polarization from abundant $\frac{1}{2}$ spin ^1H to these dilute spins ensures not only a reduction in the relaxation delay time but also a signal to noise enhancement. In CP, radiofrequency is applied to both abundant and dilute spins to achieve the Hartmann-Hahn matching condition (Hartmann and Hahn 1962, Pines, Gibby et al. 1973).

MAS may not be sufficient to average out heteronuclear dipolar coupling to abundant spins (^1H - ^{13}C , ^1H - ^{15}N). Hence, using ^1H decoupling irradiation to remove these interactions is employed, which reduces the line broadening caused by dipolar coupling and scalar coupling. Together, MAS, CP, and unique decoupling schemes form the basis to obtain high-resolution SSNMR spectra of dilute spins- ^{15}N , ^{13}C (Caravatti, Bodenhausen et al. 1982) along with enriching the biological molecules with NMR active nuclei.

1.4.2. Two-dimensional SSNMR experiments

2D experiments like dipolar assisted rotational resonance (DARR) (Takegoshi, Nakamura et al. 2001) can aid to assess the quality of the protein sample. This experiment starts with ^1H - ^{13}C CP, followed by magnetization transfer between all ^{13}C close in space (Figure 4). At short mixing times, intra-residue correlations are obtained primarily and at longer mixing times inter-residue correlations can be detected. Similarly, ^{15}N - ^{13}C correlation experiments such as 2D NCA/NCO are commonly collected for resonance assignments for $^{15}\text{N}_i$ - $^{13}\text{C}\alpha_i$ and ^{15}N - $\text{C}\alpha_{i-1}$. The transfer between ^{15}N - ^{13}C can be achieved using a heteronuclear transfer scheme such as SPECIFIC-CP (Baldus, Petkova et al. 1998)

Figure 4. Magnetization transfer pathway in the ^{13}C - ^{13}C DARR experiment. The Black dashed arrow indicates cross-polarization.

1.4.3. Three-dimensional SSNMR experiments

For sequential resonance assignments, three dimensional (3D) experiments like NCACX, NCOCX, and CANCO are acquired. In the NCACX experiment (Pauli, Baldus et al. 2001), magnetization is transferred from ^1H - ^{15}N through CP, followed by transfer to $\text{C}\alpha$ through SPECIFIC-CP. From $\text{C}\alpha$, the magnetization is transferred to ^{13}C nuclei in close space by a DARR step. At short mixing times, spin system identification is possible from intra-residue correlations. And at longer mixing times, inter-residue correlations are obtained (Figure 5). In the NCACX experiment (Pauli, Baldus et al. 2001), magnetization evolves on the ^{15}N and $^{13}\text{C}\alpha$ nuclei and is then detected on ^{13}C nuclei. NCOCX is like the NCACX experiment but in the second step, magnetization is transferred to CO through SPECIFIC-CP (Baldus, Petkova et al. 1998) (Figure 5). From this experiment $\text{N}_i\text{-CO}_{i-1}$ correlation is obtained establishing the sequential connection. CANCO experiment (Li, Berthold et al. 2007) starts with ^1H - $^{13}\text{C}\alpha$ CP followed by SPECIFIC-CP (Baldus, Petkova et al. 1998) to ^{15}N . From here magnetization is transferred to ^{13}CO using another SPECIFIC-CP (Baldus, Petkova et al. 1998). Magnetization

evolves on $^{13}\text{C}\alpha$ and ^{15}N and is detected on ^{13}CO (Figure 5). This experiment provides the through bond $\text{C}\alpha_{i+1}\text{-N}_{i+1}\text{-CO}_i$ connection though the magnetization transfers in through space. These experiments when performed at longer mixing times, structure calculation restraints can be obtained.

Figure 5. Magnetization transfer pathways of 3D SSNMR experiments used for sequential resonance assignment. The Black dashed arrow indicates cross-polarization. Gray arrow indicates magnetization transfer at longer mixing times in the NCACX experiment.

1.5. PROBING DYNAMICS USING SSNMR SPECTROSCOPY

Functional proteins do not exist as static structures, rather they undergo conformational interconversions between alternate structures around the equilibrium state (Henzler-Wildman and Kern 2007). Thus, to completely understand the biological functions of proteins, it is essential to capture the dynamic state in space and time.

SSNMR is a powerful technique to study protein dynamics that provide atomic-level insights in native environments and is sensitive to various timescales and a range of motions (Schanda and Ernst 2016). With the advancement in SSNMR methodology and pulse sequence developments, it has been possible to quantify the amplitude of protein dynamics timescales.

Using SSNMR, conformational dynamics, and plasticity in membrane proteins like β -barrel outer membrane proteins, transporters and ion-channels, viral envelopes, membrane interacting amyloid fibrils were deciphered to be compelling towards their cellular functions (Mandala, Williams et al. 2018).

Experiments specifically developed to obtain information on the site-resolved dynamics for different motional regimes are used to understand the conformational dynamics of proteins in SSNMR. Probing dynamics by NMR spectroscopy often rely on pulse sequences that cipher changes in these NMR spectroscopic parameters both in the solution and solids, as these are sensitive to the local environment, interatomic distances, bond geometry, and orientations. Observables that report on the corresponding protein dynamics and their time scales accessible to SSNMR are illustrated in Figure 6. Spin relaxations (R_1 , $R_{1\rho}$), chemical shift anisotropy (CSA), averaged dipolar couplings, quadrupolar coupling provide information on motions and amplitudes in the picosecond to micro-second and second timescales (Krushelnitsky, Reichert et al. 2013, Schanda and Ernst 2016).

Figure 6. Time scales of protein dynamics (a) and corresponding solid-state NMR observables (b) to probe these motions. Figure from (Schanda 2014).

In solutions, molecular tumbling (ps-ns timescale) averages out all the anisotropic interactions to zero, whereas, MAS is used to average out the anisotropic interactions in solids. The underlying difference is that averaging is indiscriminate in solutions, while in solids it is introduced by sample rotation (usually in μ s). By re-introducing the anisotropic interactions such that the effect of averaging by MAS is canceled, it is possible to measure the anisotropic interactions in terms of inter-nuclear distances and the direction.

Dipolar couplings (^1H - ^1H , ^1H - ^{13}C , ^1H - ^{15}N) are the predominant spin-spin interactions in SSNMR, also the direct reporter on local molecular protein dynamics in solids. Since protein conformational dynamics modulate the orientation of dipolar couplings in the presence of an external magnetic field as a function of time, measurement of ^1H - ^{13}C , ^1H - ^{15}N dipolar couplings can provide valuable information on backbone dynamics in a protein in the μ s-ns timescale.

One such experiment to obtain ^1H - ^{13}C , ^1H - ^{15}N dipolar coupling constants is the 3D dipole-chemical shift correlation experiment (DIPSHIFT) (Munowitz, Griffin et al. 1981). In this experiment, a series of 2D NCA/NCO planes are measured as a function of dipolar dephasing. Many radio-frequency schemes are available for the selective reintroduction of ^1H - ^{13}C , ^1H - ^{15}N heteronuclear dipolar couplings, whilst suppressing the homonuclear dipolar couplings (^1H - ^1H) in a fully protonated system (Schanda, Meier et al. 2011).

Averaged dipolar couplings values are extracted from the line shapes obtained by these experiments and correlated with the expected one for the rigid-limit case. Using simulation tools like SIMulation Package for SOLid-state Nmr (SIMPSON) (Bak, Rasmussen et al. 2000), the rigid-limit values for the dipolar coupling strength are calculated. The ratio of experimental and rigid-limit dipolar coupling constant ‘order parameter’- S_{NH}^2 and $S_{\text{(CH)}}^\alpha$ for ^1H - ^{15}N and ^1H - ^{13}C dipolar couplings, respectively, can be obtained site-specifically for amino acid residues in a protein. An order parameter (S) value of one reflects rigidity and zero mobility.

1.6. OBJECTIVE OF THE PRESENT WORK

This study aims to investigate the structural and dynamic properties of chromatin in physiological conditions and at atomic resolution using SSNMR spectroscopy. We primarily intend to understand the structure and dynamics of individual histones in the NCP. To date, the quantitative dynamics of histones in chromatin has not been studied. Firstly, we endeavor to probe the site-specific motions and amplitudes of globular and tail domains of human histones H4 (hH4) and H3 (hH3) in the nucleosome. In the next step, we chose to understand the influence of variations in DNA length and sequence on the core histone dynamics inferred from SSNMR studies of the so-called ‘601’-NCP (see below), and the influence on histone tail conformations.

We reconstituted human NCPs (in which either H4 or H3 was uniformly ^{13}C , ^{15}N labeled) with 145 bp “601 Widom” DNA (Lowary and Widom 1998). We chose the 601 ‘Widom’ DNA sequence of 145 bp (Lowary and Widom 1998) since it has the highest affinity offered by strong positioning to the histone octamer (Pisano, Galati et al. 2008). There are several structures available with this DNA (Vasudevan, Chua et al. 2010) for us to compare the secondary structure in our sample. We sedimented the NCPs into a well-ordered orthorhombic phase prepared by using 20 mM Mg^{2+} as previously determined by our colleagues (Berezhnoy, Liu et al. 2016) for SSNMR measurements.

We then conducted dipolar-coupling based SSNMR experiments to probe the histone globular domains for chemical shift assignment, which allows the derivation of secondary structures and comparison with the X-ray crystal structure. We then performed heteronuclear dipolar line shape measurements to map the order parameters as well as associated timescales in a site-specific manner for both histones H4 and H3 in the human NCP. Additionally, we conducted a qualitative comparison of structure and dynamics with NCP reconstituted with

human telomeric DNA repeat (TTAGGG) and a 601 'Widom' nucleosome array of 12 nucleosome repeat length (NRL) of 177 bp (12-mer array). Besides, we also conducted J-coupling based SSNMR experiments to observe the histone N-terminal tail regions in all these samples. In continuation, we endeavor to use SSNMR in studying the structure and dynamics of heterochromatin protein (HP1) binding to chromatin.

1.7. ORGANIZATION OF THE THESIS

This introduction chapter forms the basis to understand the framework of the present research work around which the thesis is organized. Chapter 2 outlines the materials used and the methodology used in the preparation of the various nucleosomes and nucleosome array. This chapter is further subdivided to include sample preparation and SSNMR experimental sections-experimental parameters, data processing, and analysis. Results and discussions are categorized into three chapters. Chapters 3 and 4 discuss the structure and dynamics of histones H4 and H3 respectively in the 601-NCP of 145 bp and their biological implications. Chapter 5 is about the extension of the structure and dynamics of histone H3 and H4 in biologically relevant samples of nucleosome array and the telomeric NCP. Chapter 6 summarizes the conclusions and significance of the research work followed by future perspectives.

Materials and methods

2. MATERIALS AND METHODS

Figure 7. Schematic illustration of the methodology used in this work. Histones and DNA were purified in *E. coli*. They were used for the reconstitution of either NCP or nucleosome array, which were packed into SSNMR rotors. Dipolar-coupling and J-coupling based experiments were used to probe the histone globular domains and tail regions respectively.

In this chapter, the methodology followed is presented. Preparation and purification of histones and DNA in *E.coli* is followed by SSNMR experiments employed for understanding the structure and dynamics of chromatin (Figure 7).

2.1. Expression and purification of human histones

NCPs reconstituted with human histones following a well-known method (Luger, Rechsteiner et al. 1999). Human histones H2A, H2B, H3, and H4 in pET-3a plasmids (courtesy Davey's Lab, NTU) were overexpressed as inclusion bodies in *E.coli* BL21 (DE3) pLysS in 2× TY media (Table 9). Four well-separated colonies were inoculated into four 5 ml aliquots of 2× TY media supplemented with appropriate antibiotics and grown at 37 °C until it became turbid. This pre-culture was combined and inoculated into a 100 ml flask with 2× TY and antibiotics and grown at 37 °C until the OD₆₀₀ reached 0.4. This starter culture was evenly distributed into twelve 500 ml baffled flasks and incubated at 37 °C shaker at 220 rpm until the OD₆₀₀ reached 0.55. At this point, the cells were induced with 0.4mM IPTG for 3 hours. Cells were harvested by centrifuging at 7000 rpm for 7 minutes at room temperature.

The cell pellet was resuspended in 100ml wash buffer (Table 8) until it became viscous. This suspension was subjected to sonication to lyse the cells until there was no more viscosity. The solution was centrifuged at 12000 rpm for 20 minutes at 4 °C. The pellet was resuspended in 150 ml wash buffer (Table 8) followed by centrifugation at 12000 rpm for 20 minutes at 4 °C. This step was repeated once followed by two more washes with wash buffer (no triton X-100). The drained pellet which contains the histone inclusion bodies was stored at -20 °C until use.

The histone inclusion body pellet was transferred into a 50 ml centrifuge tube, soaked in 1-2 ml of DMSO, and homogenized extensively with a glass rod followed by vortexing for

about 30 minutes. 40 ml of S-200 unfolding buffer (Table 8) was added and stirred gently for 1 hour at room temperature to allow maximum solubilization. The cell debris was removed by centrifugation at 12000 g for 10 minutes at 20 °C. This step was repeated until clear supernatant (containing the unfolded histones) was obtained.

The crude unfolded histone sample was loaded onto the pre-equilibrated HiPrep 26/60 Sephacryl S-200 HR column (GE Healthcare) with SAUDE-1000 buffer (Table 8), at a flow rate of 1.5-2 ml/min monitoring the elution profile at OD₂₈₀, collecting fractions of 5 ml size. Multiple runs of 10 ml sample injection were employed to load the entire sample. Eluted fractions were analyzed on 18% SDS-PAGE at 30 mA for 40-60 minutes to assess the purity.

The peak under the histone was assessed for OD₂₆₀:OD₂₈₀ and fractions containing pure histones (OD₂₆₀:OD₂₈₀ <0.7) were pooled and dialyzed against Milli-Q water with 5mM beta-mercaptoethanol at 4 °C. Post dialysis the solution was centrifuged to remove any precipitation. The concentration of histone was determined using the OD₂₇₆ values and extinction co-efficient for different histones. These were then lyophilized and stored at -80 °C. The less pure histone fractions (OD₂₆₀:OD₂₈₀ 0.7–1.2) were also pooled, followed by dialysis and lyophilization.

The less pure histones were further purified by ion-exchange chromatography to remove DNA impurities. The lyophilized histones were dissolved in SAUDE-200 buffer (Table 8), followed by centrifugation at 10000 rpm for 10 minutes to remove any insoluble matter. This was loaded onto a Resource S column (GE Healthcare) pre-equilibrated with SAUDE-200 buffer. Pure histones were eluted using a gradient elution of SAUDE-1000 buffer (Table 8) at a flow rate of 3 ml/min. The fractions were assessed for purity on 18% SDS-PAGE. Fractions containing pure histones were pooled and dialyzed against Milli-Q water at 4 °C. The concentration of histones was determined using the OD₂₇₆ values and extinction co-efficient for different histones followed by lyophilization and stored at -80 °C.

2.2. Expression and purification of ^{13}C , ^{15}N -labeled human histones for SSNMR spectroscopy

Since protein NMR spectroscopy requires isotope enriched proteins that are NMR active, the proteins are expressed in a labeled media like M9 minimal media (Table 9) providing carbon (^{13}C) and nitrogen (^{15}N) source.

To facilitate uniform ^{13}C , ^{15}N labeling of human histones H4 and H3 for SSNMR studies, the *E.coli* BL21 (DE3) pLysS cells harboring either H4 or H3 plasmids in pET-3a, (courtesy Davey's lab, NTU) were grown in 2× TY media and induced in an equal amount of M9 minimal media. The purification of ^{13}C , ^{15}N isotope-labeled human histones H4 and H3 were carried out as described in the above section.

Four well-separated colonies were inoculated into four 5 ml aliquots of 2× TY media supplemented with appropriate antibiotics and grown at 37°C. This pre-culture was combined and inoculated into a 100 ml flask with 2× TY and antibiotics and grown at 37 °C until the OD₆₀₀ reached 0.4. This starter culture was evenly distributed into four 500 ml baffled flasks (2 liters in total) and incubated at 37 °C shaker at 220 rpm until the OD₆₀₀ reached 0.55. The cells were spun down at 7000 rpm for 7 minutes at room temperature, resuspended in an equal amount (2 liters) of M9 minimal media, and continued to grow for an hour (or until the OD₆₀₀ reached 0.8) before inducing with 0.4mM IPTG for 3 hours. Cells were harvested by centrifuging at 7000 rpm for 7 minutes at room temperature. Both ^{13}C , ^{15}N isotope-labeled human histones H4 and H3 were expressed in this method and were purified as described in earlier section (2.1. Expression and purification of human histones).

2.3. Refolding histones to histone octamer (HO)

Human histones in equimolar ratios were refolded into octamers by dialysis with refolding buffer (Table 8) at 4 °C and purified by gel filtration using HiLoad 16/60 Superdex S-200 (GE healthcare). The integrity of histone octamers was assessed by 18% SDS PAGE. They were stored with 50% glycerol at -20 °C.

Lyophilized histones were dissolved in HO unfolding buffer (Table 8) to a final concentration of 2-3 mg/ml, calculated by their OD₂₇₆ values and extinction coefficients (table). The four histones- hH2A, hH2B, hH3, and ¹³C, ¹⁵N-H4 were mixed in an equimolar ratio (1:1:1:1) allowing the unfolding to proceed for 30 minutes. This was then dialyzed against the refolding buffer at 4 °C, changing the buffer twice every 5 hours. After the third buffer change, dialysis was carried out overnight.

Post dialysis, any precipitate matter was removed by centrifugation, and then refolded HO was concentrated to about 6-8 mg/ml in an Amicon concentrator device (10kDa cut off membrane). The sample was loaded onto a pre-equilibrated HiLoad 16/60 Superdex column 200 pg (GE Healthcare) with refolding buffer (Table 8) at a flow rate of 1 ml/min. Fractions were run on 18% SDS gel to analyze the integrity of HO which were then pooled and concentrated. The yield was estimated by OD₂₇₆ values. An equal volume of 100% glycerol was added to give a final concentration of 50% glycerol and stored at -20°C.

2.4. Expression and purification of 601 ‘Widom’ 145 bp DNA

The pUC19 plasmid harboring 601 ‘Widom’ nucleosome positioning sequence of 145 bp length was amplified in *E. coli* DH5α. The plasmid was purified by the alkaline lysis method. The plasmid was digested with *EcoRV*-HF. The 601-145bp DNA was separated from the vector

DNA by PEG 6000 mediated precipitation. The purity was checked by 1.5% Agarose Gel electrophoresis (Table 14) and 10% PAGE (Table 13).

About 4-5 well-separated colonies (plated from a glycerol stock of DH5 α with 601-145 bp DNA) were inoculated in 5 ml TB (Table 9) media supplemented with appropriate antibiotics and grown at 37 °C at 220 rpm until it became turbid. This was transferred into a 100 ml flask with TB medium and allowed to grow at 37 °C at 220 rpm for 2 hours. This pre-culture was equally distributed into 12 flasks (of 500ml each) containing TB medium and supplemented with antibiotics and grown at 37 °C at 220 rpm for 19 hours.

Cells were harvested by centrifugation at 7000 rpm at room temperature. The cell pellet was resuspended in alkaline lysis buffer I (Table 8) bringing the final volume to 60 ml in each centrifuge bottle. This was followed by the addition of 120 ml of alkaline lysis buffer II (Table 8) to each bottle, mixed vigorously, and incubated on ice for 20 minutes. This step was repeated thrice with 5-minute intervals. Following this, 210 ml of cold alkaline lysis buffer III (Table 8) was added gently inverting the bottles to mix and incubated on ice for 20 minutes. This was repeated every 5 minutes.

The suspension was centrifuged at 10000 g for 20 minutes at 4 °C. The supernatant was filtered through sterile gauze and 0.52 volume of isopropanol was added, swirled gently, and allowed to stand for 30 minutes, and spun at 16000g for 30 minutes at 4 °C. The pellet containing the DNA was dissolved in TE buffer (10, 50) (Table 8) and subjected to *RNaseA* digestion (10 mg/ml) overnight in a shaker at 37°C.

The DNA solution was spun down to remove any insoluble matter and transferred to Teflon tubes. This was followed by extraction with tris-saturated Phenol twice to remove proteins. The aqueous phase was extracted with Chloroform: Isoamyl alcohol (CIA) (24:1) to remove the residual phenol followed by PEG precipitation of DNA on ice with 0.2 volumes of

4M NaCl and 0.4 volumes of 40% PEG 6000 for 30 minutes. DNA was pelleted down by centrifugation at 27000g for 10 minutes at 4°C. Pellet was dissolved in TE buffer (10, 0.1) (Table 8) and extracted twice with CIA by centrifugation at 20000 g for 10 minutes at 20 °C to remove traces of PEG. To the aqueous phase, 1/10 volumes of NaCl and 2.5 volumes of 100% ice-cold ethanol was added and incubated on ice for 30 minutes. The tubes were then spun at 27000 g for 30 minutes at 4 °C. The plasmid DNA pellet was dissolved in TE buffer (10, 0.1). Concentration was estimated by OD₂₆₀ and purity by OD₂₆₀:OD₂₈₀.

2.4.1. Restriction digestion and purification

About 75-100 units of *EcoRV*-HF enzyme per mg of plasmid DNA was sufficient to carry out complete digestion. The plasmid DNA was dissolved in 1X cut smart buffer (from NEB) in which *EcoRV*-HF (also from NEB) has 100% activity. This mixture was incubated at 37°C overnight. The completion of digestion was determined by running the digested product on a 1.5% agarose gel and 10% native PAGE (Table 13).

The 601-145bp DNA was separated from vector DNA by precipitating the vector backbone with PEG-6000. PEG solution was added dropwise to the solution containing digested plasmid DNA, gradually mixing well along with 0.5M NaCl, and incubated on ice. Gradient PEG fractionation was carried out starting from the addition of 6-7% PEG-6000 at 0.5% increment until the concentration reached about 8-8.5% and 0.5M NaCl, monitoring the extent of fractionation on 1.5% FAST Gel using the FAST buffer system. In most cases, complete precipitation of the backbone was achieved at around 8.5% PEG-6000. The fractionated backbone was pelleted by centrifugation at 27000 g for 30 minutes at 4 °C. Pellet was dissolved in TE (10, 0.1) buffer, traces of PEG was removed by extracting with CIA twice with centrifugation at 27000 g for 10 minutes at 20 °C. 1/10 volumes of NaCl and 2.5 volumes of ice-cold ethanol was added to the aqueous phase and incubated on ice for 30 minutes. After

incubation, 601-145 bp DNA was pelleted down by spinning at 27000 g for 30 minutes at 4 °C. The concentration was determined by OD₂₆₀ values and the purity of the sample was assessed by OD₂₆₀/OD₂₈₀ ratio and 1.5% agarose gel and 10% PAGE using a 1×TBE buffer system (Table 8).

2.5. Expression and purification of 601 ‘Widom’ 12-mer 177 bp DNA

The 12-mer DNA was prepared using the method described by our colleagues (Chen, Yang et al. 2017). pWM530 plasmid containing 12-601-177 DNA (gift from Dr. Davey’s Lab, NTU) was amplified in *E.coli* TOP10 cells was extracted by alkaline lysis method (described in section 2.4. Expression and purification of 601 ‘Widom’ 145 bp DNA), followed by digestion with *EcoRV* enzyme to cleave the 12-601-177 DNA from the vector backbone, followed by PEG 6000 mediated fractionation. Small amounts of remaining vector backbone and purified by Sephacryl S500 gel filtration column (from GE Healthcare).

2.5.1. Restriction digestion and purification

For *EcoRV* mediated digestion, plasmid DNA was dissolved 1×NEB buffer 3.1 (50 mM Tris-HCl, 100 mM NaCl, 10 mM MgCl₂, 100 µg/mL BSA, pH 7.9) to a final concentration of 1.5 mg/mL. *EcoRV* was added as 400 units per mg of DNA. The digestion mixture was incubated at 37 °C overnight and the reaction was monitored with 0.8% agarose gel electrophoresis. Once the *EcoRV* digestion was completed, 1/10 volume of 3M sodium acetate (pH5.2) and 2.5 volumes of cold ethanol were added and incubated on ice for 30 minutes followed by centrifugation to harvest the DNA pellet. It was air-dried to remove the residual ethanol and then dissolved in 10 mL TE (10, 0.1) such that the DNA concentration was 2 mg/mL.

A stock solution of 35% PEG 6000 and 0.5M NaCl was added dropwise and the DNA solution was mixed carefully and incubated on ice for 30 minutes, followed by centrifugation at 10000 g for 30 minutes at 4 °C. The supernatant was separated and subjected to PEG precipitation repeating until the final PEG 6000 concentration reached 6.5% with 0.5% increments every time. The DNA pellets from each step were dissolved in TE (10, 0.1) buffer. The quality of the dissolved pellets and final supernatant was checked on 0.8% agarose gel. Fractions with minimal contaminations were subjected to size exclusion chromatography using the Sephacryl S500 column (from GE Healthcare).

The column was equilibrated with two-column volumes of TES9 buffer (Table 8). DNA in about 3-4 mg/mL was injected into the sample loop and eluted with TES9 buffer at a flow rate of 2 mL/min. The longer 12-mer DNA peak was eluted first, followed by the elution of short-length DNA fragments from the backbone. The fractions collected were analyzed by 0.8% agarose gel. Fractions that are found pure were pooled and concentrated three-fold with Amicon concentrator followed by precipitation with 1/10 volume of 3M sodium acetate (pH 5.2) and 2.5 volumes of 100% cold ethanol. This was incubated on ice for 30 minutes, and subject to centrifugation at 27,000 g for 30 min at 4 °C. The pellet was air-dried and dissolved in TE (10, 0.1). The DNA concentration was determined by measuring at an absorbance at 260 using a UV Nanodrop spectrophotometer and was stored at -20 °C. The remaining impure fractions were pooled and concentrated using the Amicon concentrator device (molecular weight cut-off 10 kDa) and SEC purification was repeated as described above.

2.6. Expression and purification of human telomeric DNA of 145 bp DNA

E.coli Sure 2 cells (Agilent Technologies Singapore Pte. Ltd) harboring the plasmid containing eight copies of the human telomeric repeat ATC(TTAGGG)₂₃GAT flanked by

EcoRV and *AvaI* sites (Bio Basic Asia Pacific Pte Ltd.) of 145 bp length was expressed and purified as detailed by colleagues (Soman, Liew et al. 2020).

Freshly transformed cells/ glycerol stocks were grown at 30 °C on an LB agar plate for 24 hours. Individual colonies were selected and inoculated into tubes with TB media and grown for 8 hours. 5 ml of this culture was used for a plasmid extraction using a miniprep kit (Axygen) to check the integrity of telomeric insert with restriction digestion by *EcoRV*. The clone that exhibited the highest integrity of plasmid was inoculated into 100 ml TB media containing ampicillin and chloramphenicol. It was grown for 3 hours and used as inoculum for the large-scale culture. Plasmid expression and purification were carried out using the same method described in section 2.4. Expression and purification of 601 ‘Widom’ 145 bp DNA, with an exception in growth temperature that is 30 °C and a growth period of 18 hours.

2.6.1. Restriction digestion and purification

The plasmid concentration was adjusted to 2 mg/ml in 1X cut smart buffer (from NEB) in which *EcoRV*-HF (also from NEB) has 100% activity. About 75-100 units of *EcoRV*-HF per mg of plasmid was used and incubated overnight at 37 °C for restriction digestion. The completion of digestion was determined by running the digested product on a 1.5% agarose gel and 10% PAGE.

PEG precipitation was used to separate the telomeric DNA fragment (145 bp) from the vector backbone (2700 bp). Gradient PEG fractionation at 0.25% increment was carried out to get a final concentration of 7.5% PEG and 10mM MgCl₂ until all the vector backbone was removed from the supernatant. The cloudy mixture was incubated at 42 °C for 5 minutes, followed by incubation on ice for 30 minutes. The solution was centrifuged at 27000 g for 20 minutes at 4 °C. The supernatant was run on a 10% PAGE to check the completeness of separation. The supernatant containing the blunt-ended DNA fragment was precipitated with a

3X volume of ethanol. The precipitated DNA was resuspended in TE (10, 0.1) buffer and further purified by ion exchange to remove traces of vector and recombination products.

Ion-exchange purification was carried out using a MonoQ 5/50 G1 column (GE healthcare). 2 mg of the sample was loaded onto the column at a flow rate of 0.5 ml/min. The column was washed with 10 column volumes of buffer A (Table 8). The DNA was fractioned employing a linear gradient of 40% to 65% of buffer B across 55 column volumes at flowrate 3ml/min. The fractions were run on a 10 % PAGE in 1X TBE. Fractions containing the mononucleosome DNA were combined, concentrated, and buffer exchanged to TE (10, 0.1) using a 10,000 MWCO concentrator (Millipore). This process was repeated until all the DNA was purified.

2.7. Reconstitution of the nucleosome core particle

NCP was reconstituted by salt gradient dialysis using human HO and 601-145 bp DNA (Luger, Rechsteiner et al. 1999). HO and 601-145 DNA were mixed at 2 M NaCl and the salt concentration was gradually reduced by gradient dialysis to less than 0.25 M NaCl over 20 hours. The molar ratio of octamer and DNA was determined by a small-scale titration ranging HO:DNA ratio from 0.8–1.2 with 0.1 increments of HO keeping the amount of DNA constant (0.5mg/ml). The optimum HO: DNA determined was found and used for the reconstitution of ¹³C, ¹⁵N- labeled H4- HO, 601-145bp NCP.

HO (0.631 mg/mL) and 601-145bp DNA (0.5 mg/mL) were mixed to make a molar HO:DNA ratio of 0.9:1 (determined by titration) along with 4M NaCl, 10mM DTT. This solution was mixed well and transferred into a dialysis tubing (3 kDa membrane) and gradient dialysis was started against 800 mL of RB-high buffer (Table 8) at a flow rate of 0.5 ml/min at 4 °C stirring constantly. The RB-high was constantly removed and replaced with RB-low buffer

(Table 8) by a peristaltic pump generating an exponential gradient for 20-24 hours. Following this, the sample was dialyzed against RB-low for 2-3 hours ensuring that the salt concentration reached below 0.2M NaCl. The NCPs formed were analyzed on a 6% PAGE with a 0.25× TBE buffer system (Table 8) at a constant volt of 90-100 V for 1 hour to look at nucleosomal and free DNA. The gel was pre-run for 1 hour at the same voltage conditions.

2.8. Reconstitution of nucleosomal array

The nucleosome array was reconstituted as described previously (Chen, Yang et al. 2017) using human H2A, H2B, H3, ¹³C, ¹⁵N- labeled H4 histones, and 12 tandem 177 bp repeats of the high-affinity Widom ‘601’ nucleosome positioning DNA sequence (12-mer DNA or 12-601-177 DNA). The 12-mer nucleosome array was reconstituted by stepwise salt dialysis in TEK buffer (2M KCl, 10mM Tris-HCl at pH 7.5 and 0.1mM EDTA) using HO containing ¹³C, ¹⁵N- labeled H4, and 12-mer DNA in a ratio of 0.98:1 (determined by titration). To prevent the oversaturation of DNA by the HO, competitor DNA (core length DNA from calf thymus ~150 bp) was added at 0.04:1 of competitor DNA: 12-mer DNA to the reconstitution mixture. To assess the quality of the nucleosome array, the *ScaI* restriction enzyme (NEB) digestion assay was performed in 1X cut smart buffer (NEB) for 3 hours at 37 °C, since 601-177 bp repeats are flanked by *ScaI* restriction site. The digestion products were analyzed on a 6% native PAGE and stoichiometry of histone octamer to 12-mer DNA was determined for qualitative analysis.

2.9. Reconstitution of telomeric NCP

The telomeric NCPs were reconstituted by the salt gradient dialysis method. A mixture of 20 mM Tris (pH 7.5), 2 M LiCl, 1 mM EDTA, 1 mM DTT, 4.8-7.2 μM HO, and 6 μM DNA

were added in a dialysis bag (10 kDa MWCO). The mixture was first equilibrated against high salt buffer (20 mM Tris pH 7.5, 2M LiCl, 1 mM EDTA, and 1 mM DTT) for 30 minutes at room temperature. Subsequently, a low-salt buffer (20 mM Tris pH 7.5, 1 mM EDTA, and 1 mM DTT) was slowly pumped into the dialysis buffer; meanwhile, an equal amount of the mixed buffer was withdrawn from the dialysis buffer. Overall, 1.5 L low-salt buffer was continuously pumped into 0.6 L high-salt buffer for 18 hours and then the dialysis sample was incubated in the low-salt buffer for another 4 hours. The quality of the reconstituted telomeric NCP was verified on 6% PAGE and 18% SDS PAGE.

2.10. Sample preparation for SSNMR spectroscopy

For SSNMR experiments, NCP aggregates were prepared with 20mM Mg^{2+} as previously determined by our colleagues (Berezhnoy, Liu et al. 2016). Reconstituted human NCPs in, 20 mM Tris pH 7.5, 1 mM EDTA, and 1 mM DTT (<0.25 M NaCl from gradient dialysis) were concentrated to a final concentration of 3mg/ml. 1 M $MgCl_2$ was added to make a final concentration of 1 mM Mg^{2+} ensuring thorough mixing. This was incubated at room temperature for 15 minutes and centrifuged at 10000 rpm for a minute to remove unwanted insoluble particles. Following this 1M $MgCl_2$ was added to make a final concentration of 20mM Mg^{2+} , allowed to stand at room temperature for 15 minutes. These aggregates were transferred into the 3.2mm solid-state NMR zirconia rotor (Bruker) by ultracentrifugation at 100000 g to pack the NCP with the aid of a rotor packing device (from Giotto Biotech). About 41 mg of hH4 and about 55.2 mg of hH4 labeled NCP samples were packed into a rotor. The packed rotors are stored at 4 °C until use. They are found to be stable for more than a year.

Similarly, 6 mM Mg^{2+} was used to sediment the nucleosome array in 20 mM Tris pH 7.5, 1 mM EDTA, and 1 mM DTT (<0.25 M NaCl from gradient dialysis).

Figure 8. NCP sedimented using 20mM Mg^{++} and packed into a 1.9 mm SSNMR rotor.

2.11. Sample preparation for solution NMR spectroscopy

About 0.3mM hH4 in the 601-NCP and 0.15 mM hH3 in the 601-NCP solutions in reconstitution buffer (Table 8), was exchanged with NMR buffer (Table 8) using Amicon protein concentrator with 10 kDa cut-off (from Merck Millipore). This was transferred into Norell® select series™ 3mm NMR tubes (from Merck). The quality of both the samples before and after buffer exchange was analyzed on a 6% native PAGE gel for the integrity of NCP. The NMR tubes are stored at 4 °C until use.

2.12. SSNMR experiments for ^{13}C , ^{15}N - labeled histones

SSNMR experiments were recorded at a variable temperature (VT) of -15 °C at 17.851 kHz MAS rate or a VT of -12 °C at 15.151 kHz on a 18.8 T Bruker Avance III HD standard bore spectrometer equipped with Bruker 3.2mm H/C/N Efree probe and at a VT of -15 °C at 13.3 kHz MAS rate on a 14.1 T Bruker Avance III HD wide-bore NMR spectrometer equipped with a Bruker 3.2 mm H/C/N probe. The MAS fractional heating results in an actual sample temperature of ~12 °C in all experiments.

Figure 9. Pulse sequence for ^{13}C - ^{13}C DARR

The 2D ^{13}C - ^{13}C correlation experiment serves as a signature spectrum in SSNMR. We conducted the ^{13}C - ^{13}C dipolar assisted rotational resonance (DARR) experiment, for which the pulse sequence scheme is illustrated in Figure 9. Detailed experimental parameters for various samples of histone are listed in Table 3, Table 4, Table 5, and, Table 6. To compare the histones in nucleosome array and telomeric NCP, the digitization parameters on both direct and indirect dimensions were the same as those used in the experiments performed on NCP to ensure accurate comparison of peak linewidths and intensities.

We conducted three-dimensional (3D) NCACX, NCOCX, and CANCO experiments to aid in the sequential resonance assignments of histones. The pulse sequence schemes for these experiments are illustrated in Figure 10 and the magnetization transfer pathway is explained in an earlier section (1.4.3. Three-dimensional SSNMR experiments). Detailed experimental parameters for histones H4 in the 601-NCP are listed in Table 3. Experimental parameters for histone H3 are listed in Table 4, and, Table 5. Experimental parameters for histones H4 and H3 in the telomeric NCP are listed in Table 6.

Figure 10. Pulse sequence schemes for A) NCACX/NCOCX B) CANCO.

Experiment	CC	CC	NCA	NCO	NCACX	NCOCX	CANCO
Field (T)				18.8			
MAS rate (kHz)				17.857			
Probe	Bruker 3.2 mm Efree HCN probe						
transfer 1	HC CP	HC CP	HN CP	HN CP	HN CP	HN CP	HC CP
rf field (kHz), ¹ H shape	82.2 ramp	82.2 ramp	63.7 ramp	63.7 ramp	63.7 ramp	52.1 ramp	82.2 ramp
rf field (kHz), ¹⁵ N/ ¹³ C	56.9	56.9	44.6	44.6	44.6	37.9	56.9
transfer time	1.4	1.4	1.4	1.4	1.4	1.2	0.8
carrier (ppm) ¹³ C, ¹⁵ N	103.5 –	103.5 –	– 115.8	– 115.8	– 115.8	– 115.3	55.5 –
transfer 2	DARR	DARR	NCA SPECIFIC	NCO SPECIFIC	NCA SPECIFIC	NCO SPECIFIC	CAN SPECIFIC
rf field (kHz), ¹³ C shape	–	–	30.8, tangent	48.8, tangent	30.8 tangent	45.5 tangent	31.4 tangent
rf field (kHz), ¹⁵ N	–	–	44.6	26.8	44.6	22.7	44.6
rf field (kHz), ¹ H cw	15.5	15.5	88.6	90.6	88.6	88.7	88.6
transfer time	20	100	3.5	4.5	3.5	4.5	3.5
carrier (ppm) ¹³ C, ¹⁵ N	103.5 –	103.5 –	55.5 115.8	178.5 115.8	55.5 115.8	178.0 115.3	55.5 115.8
transfer 3	–	–	–	–	DARR	DARR	NCO SPECIFIC
rf field (kHz), ¹³ C shape	–	–	–	–	–	–	48.6 tangent
rf field (kHz), ¹⁵ N	–	–	–	–	–	–	26.8
rf field (kHz), ¹ H cw	–	–	–	–	15.5	12.7	90.6
transfer time	–	–	–	–	100	100	4.5
carrier (ppm) ¹³ C, ¹⁵ N	–	–	–	–	103.5	103.0	178.5 115.8
digitalization, F1	C	C	N	N	N	N	CA
t1 increments	896	896	112	112	48	48	96
sweep width (kHz)	53571	53571	4464.25	4464.25	2976.167	3030.2	8928.5
acquisition time (ms)	8.4	8.4	12.5	12.5	8.1	7.9	5.4
digitalization, F2	C	C	C	C	CA	CO	N
t2 increments	1664	1664	512	512	96	44	48
sweep width (kHz)	53571.43	53571.43	17857.143	17857.143	8928.5	3787.466	2976.167
acquisition time (ms)	15.5	15.5	14.3	14.3	5.4	5.8	8.1
digitalization, F3	–	–	–	–	C	C	C
t3 increments	–	–	–	–	1280	1024	1280
sweep width (kHz)	–	–	–	–	53571.43	45454.547	53571.43
acquisition time (ms)	–	–	–	–	11.9	11.3	11.9
¹ H decoupling rf field (kHz) shape	80.6 SPINAL 64	80.6 SPINAL 64	80.6 SPINAL 64	80.6 SPINAL 64	80.6 SPINAL 64	75.8 SPINAL 64	80.6 SPINAL 64
total scans	48	48	196	196	96	192	64
pulse delay (s)	1.5	1.5	1.5	1.5	1.5	1.5	1.5

Table 3. SSNMR experimental parameters used for the resonance assignments of hH4 in the 601-NCP.

Experiment	CC	CC	CC	CC	NCA	NCO	NcaCX
Field (T)	18.8						
MAS rate (kHz)	15.151						
Probe	Bruker 3.2 mm Efree HCN probe						
transfer 1	HC CP	HC CP	HC CP	HC CP	HN CP	HN CP	HC CP
rf field (kHz), ¹ H	70.4	70.4	70.4	70.4	52.2	52.2	53.4
shape	ramp	ramp	ramp	ramp	ramp	ramp	ramp
rf field (kHz), ¹⁵ N/ ¹³ C	53.0	53.0	53.0	53.0	37.9	37.9	37.9
transfer time (ms)	1.0	1.0	1.0	1.0	1.0	1.0	1.0
carrier (ppm)	103.0	103.0	103.0	103.0	—	—	103.0
¹³ C, ¹⁵ N	—	—	—	—	115.4	115.4	—
transfer 2	DARR	DARR	DREAM	DREAM	NCA SPECIFIC	NCO SPECIFIC	NCA SPECIFIC
rf field (kHz), ¹³ C	—	—	11	11	28.5	45.1	28.2
shape	—	—	tangent	tangent	tangent	tangent	tangent
rf field (kHz), ¹⁵ N	—	—	—	—	37.9	22.7	37.9
rf field (kHz), ¹ H cw	14.1	14.1	76.6	76.6	89.4	89.4	89.4
transfer time (ms)	20	100	3.0	3.0	4.0	4.0	4.0
carrier (ppm)	103.0	103.0	57.0	30.0	58.0	178.0	55.0
¹³ C, ¹⁵ N	—	—	—	—	115.4	115.4	115.4
transfer 3	—	—	—	—	—	—	DARR
rf field (kHz), ¹³ C	—	—	—	—	—	—	—
rf field (kHz), ¹⁵ N	—	—	—	—	—	—	—
rf field (kHz), ¹ H	—	—	—	—	—	—	14.1
transfer time (ms)	—	—	—	—	—	—	20
carrier (ppm)	—	—	—	—	—	—	103.0
¹³ C, ¹⁵ N	—	—	—	—	—	—	—
digitalization, F1	C	C	C	C	N	N	N
t1 increments	768	768	768	768	192	192	64
sweep width (kHz)	45453	45453	45453	45453	7575.5	7575.5	3030.2
acquisition time (ms)	8.4	8.4	8.4	8.4	12.7	12.7	10.6
digitalization, F2	C	C	C	C	C	C	C
t2 increments	1536	1536	1280	1280	832	832	1216
sweep width (kHz)	53571.43	53571.43	45454.547	45454.547	29761.904	29761.904	45454.547
acquisition time (ms)	14.4	14.4	14.1	14.1	14.0	14.0	13.4
digitalization, F3	—	—	—	—	—	—	—
t3 increments	—	—	—	—	—	—	—
sweep width (kHz)	—	—	—	—	—	—	—
acquisition time (ms)	—	—	—	—	—	—	—
¹ H decoupling rf field (kHz) shape	73.5 SPINAL 64	73.5 SPINAL 64	73.5 SPINAL 64	73.5 SPINAL 64	73.5 SPINAL 64	73.5 SPINAL 64	73.5 SPINAL 64
pulse delay (s)	1.5	1.5	1.5	1.5	1.5	1.5	1.5

Table 4. SSNMR experimental parameters used for the resonance assignments of hH3 in the 601-NCP.

Experiment	NcaCX	NCACX	NCOCX	CANCO	NCACX	NCOCX
Field (T)	18.8					
MAS rate (kHz)	15.151					
transfer 1	HC CP	HN CP	HN CP	HC CP	HN CP	HN CP
rf field (kHz), ¹ H	53.4	52.2	52.2	70.0	52.2	52.2
shape	ramp	ramp	ramp	ramp	ramp	ramp
rf field (kHz), ¹⁵ N/ ¹³ C	37.9	37.9	37.9	53.0	37.9	37.9
transfer time	1.0	1.0	1.0	0.8	1.0	1.0
carrier (ppm)	103.0	—	—	55.0	—	—
¹³ C, ¹⁵ N	—	115.4	115.4	—	115.4	115.4
transfer 2	NCA	NCA	NCO	CAN	NCA	NCO
	SPECIFIC	SPECIFIC	SPECIFIC	SPECIFIC	SPECIFIC	SPECIFIC
rf field (kHz), ¹³ C	28.2	28.5	45.1	28.9	28.5	45.1
shape	tangent	tangent	tangent	tangent	tangent	tangent
rf field (kHz), ¹⁵ N	37.9	37.9	22.7	37.9	37.9	22.7
rf field (kHz), ¹ H cw	89.4	89.4	89.4	89.4	89.4	89.4
transfer time	4.0	4.0	4.0	3.0	4.0	4.0
carrier (ppm)	55.0	55.0	178.0	55.0	55.0	178.0
¹³ C, ¹⁵ N	115.4	115.4	115.4	115.4	115.4	115.4
transfer 3	DARR	DARR	DARR	NCO SPECIFIC	DARR	DARR
rf field (kHz), ¹³ C	—	—	—	45.0	—	—
rf field (kHz), ¹⁵ N	—	—	—	22.7	—	—
rf field (kHz), ¹ H	14.1	14.1	14.1	89.4	14.1	14.1
transfer time	100	75	75	3.0	20	20
carrier (ppm)	103.0	103.0	103.0	178.0	103.0	103.0
¹³ C, ¹⁵ N	—	—	—	115.4	—	—
digitalization, F1	N	N	N	CA	N	N
t1 increments	64	48	48	84	48	48
sweep width (kHz)	3030.2	3030.2	3030.2	7575.5	3030.2	3030.2
acquisition time (ms)	10.6	7.9	7.9	5.5	7.9	7.9
digitalization, F2	C	CA	CO	N	CA	CO
t2 increments	1216	84	36	48	84	36
sweep width (kHz)	45454.547	7575.864	3030.2	3030.2	7575.864	3030.2
acquisition time (ms)	13.4	5.5	5.9	7.9	5.5	5.9
digitalization, F3	—	C	C	CO	C	C
t3 increments	—	1088	1088	1088	1088	1088
sweep width (kHz)	—	45454.547	45454.547	45454.547	45454.547	45454.547
acquisition time (ms)	—	12.0	12.0	11.9	12.0	12.0
¹ H decoupling	73.5	73.5	73.5	73.5	73.5	73.5
rf field (kHz)	SPINAL 64	SPINAL 64	SPINAL 64	SPINAL 64	SPINAL 64	SPINAL 64
shape						
pulse delay (s)	1.5	1.5	1.5	1.5	1.5	1.5

Table 5. SSNMR experimental parameters used for the resonance assignments of hH3 in the 601- NCP

(continued)

Field (T)	18.8					
Sample	Telomeric NCP (¹³ C, ¹⁵ N hH4)			Telomeric NCP (¹³ C, ¹⁵ N hH3)		
Experiment	CC	NCA	NCO	CC	NCA	NCO
MAS rate (kHz)	17.857					
Probe	1.9 mm HCN MAS probe					
transfer 1	HC CP	HN CP	HN CP	HC CP	HN CP	HN CP
rf field (kHz), ¹H	81.5	76.9	76.9	67.4	64.9	64.9
shape	ramp	ramp	ramp	ramp	ramp	ramp
rf field (kHz), ¹⁵N/¹³C	62.5	44.6	44.6	53.0	53.0	53.0
transfer time	1.2	1.0	1.0	1.0	1.2	1.2
carrier (ppm)	—	—	—	—	—	—
¹³C, ¹⁵N	102.6	115.0	115.0	102.7	120.1	120.1
transfer 2	DARR	NCA	NCO	DARR	NCA	NCO
		SPECIFIC	SPECIFIC		SPECIFIC	SPECIFIC
rf field (kHz), ¹³C	—	31.7	51.8	—	27.2	43.9
shape	—	tangent	tangent	—	tangent	tangent
rf field (kHz), ¹⁵N	—	—	—	—	37.9	22.7
rf field (kHz), ¹H cw	16.1	96.6	91.8	15.1	93.6	91.8
transfer time	20	3.5	4.0	20	3.0	3.5
carrier (ppm)	102.6	54.6	177.6	102.7	54.7	177.8
¹³C, ¹⁵N	—	115.0	118	—	120.1	120.1
transfer 3	—	—	—	—	—	—
rf field (kHz), ¹³C	—	—	—	—	—	—
shape	—	—	—	—	—	—
rf field (kHz), ¹⁵N	—	—	—	—	—	—
rf field (kHz), ¹H cw	—	—	—	—	—	—
transfer time	—	—	—	—	—	—
carrier (ppm)	—	—	—	—	—	—
¹³C, ¹⁵N	—	—	—	—	—	—
digitalization, F1	C	N	N	C	N	N
t1 increments	896	88	72	768	76	76
sweep width (kHz)	53571	3571.4	2976.167	45453	3030.2	3030.2
acquisition time (ms)	8.4	12.3	12.1	8.4	12.5	12.5
digitalization, F2	C	C	C	C	C	C
t2 increments	1664	1536	512	1536	448	448
sweep width (kHz)	53571.43	53571.43	17857.143	53571.43	15151.516	15151.516
acquisition time (ms)	15.5	14.3	14.3	14.4	14.8	14.8
digitalization, F3	—	—	—	—	—	—
t3 increments	—	—	—	—	—	—
sweep width (kHz)	—	—	—	—	—	—
acquisition time (ms)	—	—	—	—	—	—
¹H decoupling	80.6	80.6	80.6	81.4	81.4	81.4
rf field (kHz)	SPINAL 64	SPINAL 64	SPINAL 64	SPINAL 64	SPINAL 64	SPINAL 64
shape						
pulse delay (s)	1.5	1.5	1.5	1.5	1.5	1.5

Table 6. SSNMR experimental parameters for hH4 and hH3 in telomeric NCPs

2.12.1. SSNMR experiments to characterize site-specific dynamics

SSNMR experiments were performed to characterize site-specific dynamics for histone hH4 and hH3 in the 601-NCP. ^1H - ^{15}N and ^1H - ^{13}C dipolar line shapes were measured by using 3D dipole-chemical shift correlation experiments (DIPSHIFT) (Munowitz, Griffin et al. 1981), where the dipolar line shapes are extracted from the indirect recoupling dimension and the chemical sites are identified from the NCA correlations. The R-symmetry sequence, R12_1^4 (Levitt 2002), is implemented for ^1H - ^{15}N and ^1H - ^{13}C dipolar recoupling in the 3D DIPSHIFT experiments. Dipolar line shapes were extracted unambiguously and simulated using SIMPSON (Bak, Rasmussen et al. 2000) to obtain the dynamically averaged dipolar coupling values and determine the order parameter (S) in the ns– μs timescale. An S value of one—reflects rigidity and, zero—the mobility for a given amino-acid residue. 3D DIPSHIFT experiments were conducted at 15.151 kHz, and the NCA transfer was achieved by 4 ms SPECIFIC-CP. The ^1H - ^{15}N and ^1H - ^{13}C dipolar coupling were reintroduced by applying R12_1^4 symmetry sequences on the ^1H channel in a constant-time manner.

The μs –ms dynamics are typically observed as correlated motions in larger protein segments or domains and might mediate a variety of biological functions. Herein, we investigate the dynamics in this timescale for both hH4 and hH3 in the NCP based on the cross-peak intensities in the 3D CANCO spectrum. Normalized cross-peak intensities in SSNMR dipolar-transfer based heteronuclear correlation spectrum such as CANCO, CONCA and NCA reflect protein internal mobility on the μs –ms timescale because such motions interfere with relaxation rates ($T1\rho$, $T2^*$) during CP transfer periods and chemical shift evolution.

2.12.2. SSNMR experiments to characterize the tail domains

The scalar coupling –based INEPT (insensitive nuclei enhanced by polarization transfer) experiment is used to observe highly flexible regions of proteins that have longer transverse relaxation (T_2) values compared to the well-structured regions of the same protein. The signal from the rigid portions with shorter ^1H T_2 decays during the INEPT delays, leaving observable signals only from the segments with mobility on the ns or shorter timescale (Morris and Freeman 1979, Morris 1980).

2D INEPT experiments were recorded on a 14.1 T Bruker Avance III HD wide-bore NMR spectrometer equipped with a Bruker 1.9 mm H/C/N probe at 35 kHz MAS rate for histone hH4 in the 601-NCP, nucleosome array, and telomeric NCP. For hH3 in the 601-NCP and telomeric NCP, the experiments were recorded on a 18.8 T Bruker Avance III HD standard bore spectrometer equipped with Bruker 3.2mm H/C/N Efree probe at 15.151 kHz MAS. ^1H - ^{13}C and ^1H - ^{15}N correlation spectra containing signals from the flexible N-terminal tails of histone hH4 and hH3 in all these samples were obtained. The total duration of ^1H - ^{13}C and ^1H - ^{15}N magnetization transfer periods were 1ms and 1.4ms, respectively. The sample temperature was maintained $\sim 12^\circ\text{C}$ for all experiments. Typical ^1H , ^{13}C , and ^{15}N 90° pulse lengths were 2.5, 3.6, and 5.2 μs , respectively, and proton decoupling was applied during the chemical shift evolution periods for hH3 in the 601-NCP and telomeric NCP. For hH4 in the 601-NCP and 12-mer array 2.15, 2.6, and 3.7 μs 90° pulse lengths were used for ^1H , ^{13}C , and ^{15}N respectively.

2.13. SSNMR data processing

The data was processed using NMRpipe (Delaglio, Grzesiek et al. 1995) and sequential resonance assignment was carried out using Sparky (Lee, Tonelli et al. 2015) and CCPNMR-Analysis (Stevens, Fogh et al. 2011). ^{13}C chemical shifts were referenced with adamantane

using DSS scale (downfield signal at 40.48 ppm) and ^{15}N chemical shift was indirectly calculated. The processing parameters for histones hH4 and hH3 in the NCP are described below.

For hH4 in the NCP, 2D CC DARR data were processed with 40 Hz Lorentzian-to-Gaussian and 60° -shifted sine-bell window functions in both dimensions. Zero-filling to 2048 and 1024 points were applied to F2 and F1 dimensions, respectively. 2D NCA and NCO data were processed with 30 Hz. Lorentzian-to-Gaussian and 60° -shifted sine-bell squared window functions in both dimensions. Zero-filling to 2048 and 256 points were applied to F2 and F1 dimensions, respectively. 3D NCACX, NCOCX, and CANCO data were processed with 60 Hz Lorentzian-to-Gaussian and 60° -shifted sine-bell squared window functions in all three dimensions. Zero-filling to 2048, 256, and 256 points were applied to F3, F2, and F1 dimension, respectively

For hH3 in the NCP, apodization applied in the 2D CC DARR, CC DREAM data were 40 Hz Lorentzian-to-Gaussian and 60° -shifted sine-bell window functions in both dimensions. Zero-filling to 3072 and 2048 points were applied to F2 and F1 dimensions, respectively. 2D NCA and NCO data were processed with 40 Hz Lorentzian- to-Gaussian and 60° -shifted sine-bell squared window functions in both dimensions, and zero-filling to 2048 and 512 points in the F2 and F1 dimension, respectively. 3D NCACX, NCOCX, and CANCO data were processed with 60 Hz Lorentzian-to-Gaussian and 60° -shifted sine-bell squared window functions in all three dimensions. Zero-filling to 4096, 256, and 256 points were applied to F3, F2, and F1 dimensions, respectively.

^1H - ^{15}N and ^1H - ^{13}C dipolar coupling line shapes were extracted from the 3D DIPSHIFT experiment and fitted by simulations in SIMPSON (Bak, Rasmussen et al. 2000). The line shapes in the frequency domain were obtained via Fourier Transform of the experimental

dipolar coupling trajectories with zero-filling to 256 points. The regions of -2–2 kHz of the ^1H - ^{15}N dipolar line shapes and -4–4 kHz of the ^1H - ^{13}C dipolar line shapes were considered in the fitting.

2.14. SSNMR based sequential resonance assignment

The basic principle and approach of usage of multidimensional NMR experiments for sequential assignments are the same in solution-NMR and SSNMR. 2D ^{13}C - ^{13}C correlation DARR spectra recorded at different mixing times (for example, 20ms, 100ms, 250ms, etc.) serve as a signature spectrum. The high-resolution 2D ^{13}C - ^{13}C DARR spectrum at a short mixing time that displays well-dispersed peaks can be used as a starting point to identify intra-residue correlations. Different amino acid types can be identified based on the distinct correlation patterns they exhibit and their unique chemical shifts (chemical shift statistics from BMRB: www.bmrb.wisc.edu). In this way, several amino acid types can be identified. For example, all the 7 threonines, 6 alanines, and 1 serine were identified for histone H4 in the 601-NCP (Figure 11). This also served as starting points for sequential resonance assignments. At longer mixing times, inter-residue correlations can be observed.

3D experiments- NCACX, NCOCX, and CANCO experiments were obtained at a ^{13}C - ^{13}C DARR mixing period of 100ms. These spectra were used for sequential resonance assignments of histone globular domains in the 601 ‘Widom’ NCP. This is done by a sequential back-bone walk through the ^{15}N dimension of a given spin system namely-*i* in the spectra of NCACX to obtain intra residue correlations. From here the correlation between $\text{C}\alpha_i$ - CO_{i-1} in the N_i dimension of CANCO is obtained. The correlation of this CO_{i-1} to the diagonal peak in N_i dimension of NCOCX gives the corresponding cross-peaks of CO_{i-1} like $\text{C}\alpha_{i-1}$ and side-chain carbons. With this, we have established the sequential connectivity between *i* and *i*-1. The next

step is to identify the N_{i-1} and continue the walk until we obtain $i-2, i-3, \dots, i-n$ connectivity (Figure 12). When a sequential backbone walk is obstructed by missing peaks, a new spin system can be selected to start another backbone walk. The sequential backbone walk is visualized with strip plots in the ^{15}N dimension which connects residues in a sequence through backbone resonances.

Figure 11. 2D ^{13}C - ^{13}C correlation DARR spectrum of H4 in NCP with few intra residue correlations. Intra residue correlations of all the 7 threonines, a serine are marked. All the 6 alanine correlations are indicated in the box.

Figure 12. Illustration of SSNMR based sequential resonance assignment strategy using 3D NCACX, CANCO, and NCOCX.

2.15. SSNMR chemical shift-based secondary structure prediction

Secondary structures such as α -helices, β -strands, and coils are commonly used to describe, and visualize protein tertiary structures (Yang, Gao et al. 2018). Chemical shifts are the most accurately measurable NMR parameters. They are sensitive to the molecular structure and chemical environment. Amino acids have unique chemical shifts due to the distinct chemical groups they possess, and these are further affected by the secondary structure in the protein. Most often they are used in the prediction of secondary structure in native and/or non-native states of a protein (Mielke and Krishnan 2009).

Various tools and webservers precisely predict and identify the secondary structures based on backbone chemical shifts and protein sequences. Some of them are, the secondary chemical shift ($\Delta\delta$) values (Luca, Filippov et al. 2001) and the chemical shift index (CSI) (Hafsa, Arndt et al. 2015), and the TALOS-N –derived secondary structure probability (Shen, Delaglio et al. 2009). SSNMR chemical shifts (both backbone and side-chain) obtained from resonance assignments are used in the prediction of secondary structures. CSI 3.0 uses backbone NMR chemical shifts ($C\alpha$, CO , $C\beta$, N , $H\alpha$, NH) and sequence data to identify α

helices, β strands, β turns (type I, II, VIII, I', and II'), coil regions, β hairpins, interior β strands, and edge β strands. (Hafsa, Arndt et al. 2015). Before the development of such tools, it was a pre-requisite to obtaining a complete sequential assignment to obtain secondary structures. Another tool, TALOS-N, is an artificial neural network (ANN) based hybrid system for empirical prediction of protein backbone ϕ/ψ torsion angles, sidechain χ_1 torsion angles, and secondary structure using a combination of six kinds (HN, H α , C α , C β , CO, N) of chemical shift assignments for a given residue sequence (Shen, Delaglio et al. 2009). Following the sequential assignment, we used TALOS-N to predict the secondary structure of histones in the 601-NCP.

Results and Discussion: Structure and Dynamics of Histone H4 in the NCP

3.1. HISTONE H4

The histone H4 in the chromatin achieves two important biological functions. Firstly, the interaction of the H4 N-terminal tail with the acidic patch formed by the basic amino acids in the H2A-H2B dimer of the neighboring nucleosome mediates fiber compaction, which is the most basic level of compaction (Kalashnikova, Porter-Goff et al. 2013). Mutational disruption of these N-terminal residues has been reported to cause a structural disorder that inhibits the chromatin compaction (Zhou, Feng et al. 2012). Secondly, PTMs of residues in the H4 N-terminal tail is the basis of recognition in the DNA double-strand break repair (DSB) (Bird, Yu et al. 2002, Hunt, Ramnarain et al. 2013, Dhar, Gursoy-Yuzugullu et al. 2017). Several other biological functions are mediated through H4 together with other histones. For example, various PTMs on H4 in the CENP-A (a variant of histone H3 in centromere) containing nucleosomes are required for proper kinetochore assembly and centromere specification (Fukagawa 2017). A recent study has demonstrated the interaction of H4 tail in the nucleosome with HIV-1 integrase is crucial for the integration of viral DNA into the host genome, which could be a potential therapeutic target in the treatment of viral diseases (Benleulmi, Matysiak et al. 2017). The importance of histone PTMs both on the globular and tail domains was previously highlighted (in section 1. Introduction). While PTMs on the H4 N-terminal tail were extensively studied, similar studies are limited for the H4 globular domain that also contributes to the regulation of nucleosome structure and dynamics (Mersfelder and Parthun 2006, Tessarz and Kouzarides 2014).

We begin with investigations to understand the structure and map the site-specific dynamics of individual histones in nucleosomes. Dipolar-coupling based SSNMR experiments to obtain chemical shift assignments were conducted and obtain information on the secondary structure for the globular domain of ^{13}C , ^{15}N - labeled histone H4 in the 145 bp NCP with 601

‘Widom’ DNA. This was followed by experiments to obtain site-specific motions and amplitudes. J-coupling based experiments were used to characterize the N-terminal tails of H4. The 601 ‘Widom’ DNA of 145 bp (Lowary and Widom 1998) was chosen for this study. The 601 ‘Widom’ DNA the highest known affinity to the histone octamer (HO) and is the sequence used in many nucleosomes having the crystal structures solved (Vasudevan, Chua et al. 2010), which makes it advantageous for comprehending the structure and dynamics of nucleosome arrays and NCPs reconstituted with natural DNA sequences at physiological conditions. In this chapter, the structure and dynamic characterization of histone H4 in the 601-NCP using SSNMR is discussed.

3.2. SAMPLE QUALITY

Human histones including the ^{13}C , ^{15}N - labeled hH4 were purified using the method described in previous sections (2.1. Expression and purification of human histones) and were analyzed on a 18% SDS PAGE (Figure 50). The quality of the purified DNA used in this study, 601 ‘Widom’ DNA of 145 bp, was evaluated on 1.5% agarose gel (Figure 54). Both histones and DNA were pure. Those histones were refolded into HO that was verified to be intact (left panel in Figure 51) and reconstituted into NCP together with the DNA. The optimum HO:DNA molar ratio was found to be 0.9 for the reconstitution of the 601 ‘Widom’ NCP. This was determined by a small-scale titration with the HO:DNA molar ratio ranging from 0.8–1.2 and a constant DNA concentration (0.5 mg/ml) (Figure 13). The quality of the NCP containing the ^{13}C , ^{15}N - labeled hH4 was excellent as evidenced by the integrity of individual histones on 18% SDS-PAGE (Figure 14), and the nucleosomal DNA on 6% native PAGE (right panel in Figure 14). Sedimented NCP packed into a SSNMR rotor was a gel-like precipitate with about 50% hydration as determined by ^1H NMR spectrum. The concentration is about 500 mg/ml, which

falls within the range of the *in vivo* heterochromatin concentration (Bohrmann, Haider et al. 1993, Weidemann, Wachsmuth et al. 2003, Bertin, Mangenot et al. 2007).

Figure 13. Reconstitution of 601 'Widom' NCP with ^{13}C , ^{15}N -labeled H4 analyzed on 6% native PAGE. Small-scale titration of HO:DNA molar ratios ranging from 0.8-12.

Figure 14. The integrity of histones in reconstituted NCP analyzed on 18% PAGE. Individual histones (including ^{13}C , ^{15}N -H4) were assessed in both histone octamer (left) and NCP (right).

3.3. SSNMR STUDIES OF HISTONE H4 GLOBULAR DOMAIN IN THE NCP

3.3.1. Chemical shift assignment

Figure 15. 1D ^1H - ^{13}C CP SSNMR spectrum of histone H4 in the Mg^{2+} sedimented 601 'Widom' NCP with chemical shift ranges of protein carbons. 1D was collected using CP at 17.851 kHz MAS on a 18.8 T Bruker Avance III HD standard bore spectrometer equipped with a Bruker 3.2 mm H/C/N Efree probe.

All SSNMR experiments were collected using CP to enhance the signal to noise (S/N) of dilute $\frac{1}{2}$ spin nuclei (^{13}C , ^{15}N). A sample temperature of $\sim 12^\circ\text{C}$ was used for all the SSNMR experiments (Figure 56). The one-dimensional (1D) ^{13}C spectrum of histone H4 in the Mg^{2+} sedimented NCP is shown in Figure 15. The quality of the spectrum is excellent indicated by the high resolution of the ^{13}C peaks of the folded protein.

The dipolar-coupling based two-dimensional (2D) dipolar assisted rotational resonance (DARR), NCA, NCO, and three-dimensional (3D) NCACX, NCOCX, CANCO SSNMR experiments were conducted to facilitate sequential resonance assignments for the hH4 in the 601 'Widom' NCP. The NCP is a ~ 200 kDa complex, of which ^{13}C , ^{15}N -labeled hH4 constitutes 12% by weight. The NCP sample is highly homogenous as manifested by the outstanding resolution of ^{13}C , ^{15}N -labeled hH4 in the DARR spectrum (Figure 18). By sequential walk

through the 3D experiments (representative sequential walk for residues are shown in Figure 16), it was possible to achieve near-complete resonance assignment of the globular domain of hH4 in the NCP (N25-G101) as illustrated in Figure 17. The N-terminal tail region of hH4 (S1-D24) is not detectable in these dipolar-coupling based experiments, due to decreased dipolar couplings in these regions owing to its dynamic nature. We will use different experiments to observe them in the same sample (3.4. SSNMR studies of the histone H4 tail region in the NCP). The complete chemical shift assignment is deposited on the BioMagResBank (BMRB: www.bmrb.wisc.edu) (Ulrich, Akutsu et al. 2008, Romero, Kobayashi et al. 2020) site under accession number 27457.

Figure 16. SSNMR based sequential resonance assignment of residues E63–T73 of hH4 in the NCP. Strip plots of 3D NCACX (red), NCOCX (blue) and CANCO (black) spectra used for the sequential assignment walk are shown for residues E63–T73. The sequential connections are shown by dash lines. The peak alignments of $N_iCA_iCO_i-N_{i+1}CO_iCO_i-CA_{i+1}N_{i+1}CO_i$ and $N_iCA_iCX_i-N_{i+1}CO_iCX_i$ are indicated by black dash lines and the peak alignments of $N_{i+1}CO_iCX_{i+1}-N_{i+1}CA_{i+1}CX_{i+1}$ are indicated by grey dash lines.

Figure 17. Sequential assignment graph showing carbon and nitrogen spins of hH4 N25–G102. The assigned and unassigned spins are shown in black and grey circles, respectively.

Figure 18. 2D ^{13}C – ^{13}C DARR spectrum of hH4 in the Mg^{2+} sedimented 601 ‘Widom’ NCP with representative resonance assignment. Data was collected with 100 ms DARR mixing time.

3.3.2. Secondary structure

In the next step, we present the secondary chemical shift values ($\Delta\delta$) (Luca, Filippov et al. 2001) values and the chemical shift index (CSI) (Hafsa, Arndt et al. 2015) and TALOS-N derived secondary structure probability (Shen, Delaglio et al. 2009) (Figure 19 B-D respectively). The comparison of TALOS-N based secondary structure of the globular domain of hH4 in the Mg^{2+} sedimented NCP concurs well with the secondary structure derived from X-ray crystallography (PDB ID- 1KXI) illustrated in Figure 19 D and E. The hH4 in the sedimented NCP show all the characteristic features of the of three α -helices $\alpha 1$, $\alpha 2$, and $\alpha 3$ linked by the L1 and L2 loops. Overall, these structural analyses indicate that the majority of

the hH4 form continuous helices constituted by K31 – R40, I50 – H75, and A83 – R92 which are connected by the loop regions. Three short stretches of amino acids R45 – S47, R79 – V81, and R95 – Y98 form the β -strands. The unambiguous chemical shift assignment of the hH4 globular domain backbone resonances will facilitate the future study of the structure and internal mobility of hH4 in nucleosome arrays, chromatin fibers, and NCP constructed with natural DNA sequences.

Figure 19. Illustration of comparison of NMR based secondary structure prediction with that of X-ray crystallography (from PDB structure 3LZ0 (Vasudevan, Chua et al. 2010)). (A) The sequence of hH4 protein. (B) Secondary ^{13}C chemical shift ($\Delta\delta\text{C}$) plot, (C) CSI plot, and (D) TALOS-N structure prediction for hH4 in NCP. Asterisk marks the residues having C β unassigned. (E) Schematic secondary structure representation of hH4 X-ray structure in NCP PDB ID: 3LZ0. Reprinted from (Shi, Prasanna et al. 2018). Copyright Wiley-VCH.

3.3.3. Dynamics in the ns- μ s timescale

The site-specific dynamics for hH4 in the NCP were elucidated using the 3D-DIPSHIFT (Munowitz, Griffin et al. 1981) experiment with the implementation of the R12₁⁴ symmetry sequence (Levitt 2002). 50 uniquely resolved peaks were observed for hH4 in the NCP in the 2D NCA spectrum. The ¹H-¹⁵N and ¹H-¹³C dipolar line shapes were unambiguously extracted from the indirect recoupling dimension identifying the sites from the NCA correlations. These were fitted to obtain the dynamically averaged dipolar coupling values. Experimental dipolar line shape fits are shown in Figure 20 and Figure 21. The order parameters, S_{NH} and $S_{(CH)\alpha}$ obtained (ratio of experimental dipolar coupling constants to values in the rigid limit) for ¹H-¹⁵N and ¹H-¹³C are summarized for individual residues in Figure 22A and mapped onto the H4 X-ray structure PDB:3LZ0 (Vasudevan, Chua et al. 2010) (Figure 22B and C). The HC and HN dipolar coupling constants obtained are indicated in Table 15. Overall, the absence of large-amplitude motions in the ns- μ s timescale across the globular domain of hH4 (0.8-1, particularly for G28-R95) in the NCP signifies that the amino acids are compactly packed and well ordered.

The amino acid stretch T96-G102 in the C-terminal region of histone H4 exhibits large-amplitude motions in the ns- μ s timescale. A previous study has shown that proper interaction of the H4 C-terminal tail within the histone octamer mediates nucleosome stability. Any modifications in the conformational flexibility of the H4 C-terminal tail greatly alters the stability of histone octamer and nucleosome remodeling (Flaus, Rencurel et al. 2004, Chavez, Scorgie et al. 2012). Motions in this timescale are characteristic of local structural features-side-chain motions and loop motions. The dynamics of hH4 in 601 NCP in the ns- μ s timescale shows that the globular domain is compactly packed, except the C-terminal stretch.

Figure 20. Site-resolved experimental (dotted) and simulated (solid) ^1H - ^{15}N dipolar line shapes for hH4 protein in the NCP. The experimental line shapes are extracted from a 3D DIPSHIFT with R12,⁴ symmetry dipolar recoupling pulses. Reprinted from (Shi, Prasanna et al. 2018). Copyright Wiley-VCH.

Figure 21. Site-resolved experimental (dotted) and simulated (solid) ^1H - $^{13}\text{C}\alpha$ dipolar line shapes for hH4 protein in the NCP. The experimental line shapes are extracted from a 3D DIPSHIFT with R12,⁴ symmetry dipolar recoupling pulses. Reprinted from (Shi, Prasanna et al. 2018). Copyright Wiley-VCH.

Figure 22. Site-specific dynamics of hH4 in the ns- μ s timescale. (A) Order parameters, $S_{(CH)\alpha}$ and S_{NH} , were obtained by fitting 1H - $^{13}C_\alpha$ and 1H - ^{15}N dipolar line shapes extracted from 3D DIPSHIFT SSNMR experiments. (B) $S_{(CH)\alpha}$ mapped onto the H4 structure with PDB:3LZ0 (Vasudevan, Chua et al. 2010). (C) S_{NH} mapped onto the H4 structure (PDB: 3LZ0 (Davey, Sargent et al. 2002)). Residues of hH4 having no site-resolved order parameters due to peak overlap and the flexible tails are shown with cyan ribbon. Reprinted from (Shi, Prasanna et al. 2018). Copyright Wiley-VCH.

3.3.4. Dynamics in the μ s-ms timescale

Normalized cross peak intensities in the 3D CANCO spectrum (described in page 41) which are used to determine the sequential connection from i to $i+1$ residue, can also be used in interpreting the site-specific protein motions in the μ s-ms timescale. These are CP based experiments involving heteronuclear dipolar magnetization transfer. Motion in the μ s-ms timescale would result in perturbations in the relaxation rates ($T1\rho$, $T2^*$), therefore changes in the CANCO peak intensities. Site-resolved CANCO peak intensities of the globular domain of hH4 in the NCP are resolved in Figure 23A and mapped on to the X-ray structure PDB ID- 3LZ0 (Vasudevan, Chua et al. 2010) in Figure 23B.

Four stretches of amino acids display normalized CANCO cross-peak intensities smaller than 0.4, suggesting pronounced motions in the μ s-ms timescales in these regions, G28–P32, G41–Y51, D85–A89, and T96–G102 highlighted in Figure 23C. The high mobility of the region G28–P32 in hH4 can be attributed to the proximity to the N-terminal tail region which is highly mobile. A part of region G41–Y51 in H4, (G41–G48) contacts the nucleosomal DNA at the superhelical location (SHL) ± 0.5 . This region is also close to the nucleosome dyad. While the region L49–Y51 is adjoining the stretch on the solute accessible face of the nucleosome. This region contributes to the dynamic interface of the HO with DNA, possibly facilitating nucleosome unwrapping and DNA accessibility. The stretch of D85–A89 in the $\alpha 3$ helix exhibit less restrictive motions, Y88 from this stretch participates in H2B–H4 interaction and is also reported to undergo PTM (Tan, Luo et al. 2011). Relatively enhanced dynamics are observed in the region T96–Y98 in hH4; this can be attributed to the direct interaction with the H2A C-terminus facilitating nucleosome mobility and sliding (Flaus, Rencurel et al. 2004). It is also interesting that some of the residues in these regions in the H4 globular domain that show enhanced mobility in μ s-ms timescales are also targets for various PTMs and regulation

of chromatin structure (Mersfelder and Parthun 2006). The region of E52–R55 shows the relatively most restricted motions in hH4, owing to the participation in a complex hydrogen bond network. Motions in the $\alpha 1$, $\alpha 2$ and L2 regions of hH4 are relatively constricted, which may contribute to the stability of the octameric core in the nucleosome.

Overall, we have shown the internal motions that exist in the globular domain of hH4 in 601-NCP in the μ s-ms timescales.

3.4. SSNMR STUDIES OF THE HISTONE H4 TAIL REGION IN THE NCP

We obtained 2D ^{13}C - ^1H and ^{15}N - ^1H correlation spectra using the INEPT pulse scheme that is based on J-coupling. The N-terminal tail domains of hH4 in the NCP are highly dynamic and are thus observable in these experiments, while absent in the dipolar-coupling based SSNMR experiments. The resonances obtained agree well with the previous SSNMR study of ^{13}C , ^{15}N - labeled H4 in the 17 mer nucleosome array (Gao, Nadaud et al. 2013). The spin system type could be identified based on the characteristic chemical shifts of amino acids. Sequential resonance assignment based on INEPT experiments was not feasible due to insufficient resolution and severe resonance overlapping.

Hence, we used solution-state NMR to characterize the tail domains. Using 3D solution NMR spectra, HNCA, HNCOCA, HNCACO, and NOESY-HSQC and HCCH-TOCSY, we carried out sequential resonance assignments.

Figure 23. Site-specific dynamics of hH4 in the μ s-ms timescale. (A) Normalized peak intensities extracted from the 3D CANCO spectrum. Red box-regions with <0.4 CANCO peak intensities). Error bars are derived from the RMSD of noise in the spectrum. A horizontal line is drawn to guide visualization. (B) Normalized CANCO peak intensities mapped onto hH4 structure PDB: 3LZ0 (Vasudevan, Chua et al. 2010). Residues of hH4 having no site-resolved CANCO peaks due to overlap and the flexible tails are shown with cyan ribbon. (C) Regions in red-box highlighted on the hH4 structure PDB: 3LZ0 (Vasudevan, Chua et al. 2010). Adapted from (Shi, Prasanna et al. 2018). Copyright Wiley-VCH.

About 80%–17 out of 24 residues in the N-terminal tail region of hH4 (S1-D24) in the NCP were assigned and illustrated for several residues on the 2D ^1H - ^{15}N HSQC and ^1H - ^{13}C HSQC spectra (upper and lower left panel of Figure 24). The majority of the backbone and the side chain NMR resonances of glycine and lysine fall into a very narrow range due to the disordered structural features. Particularly, the resonances of three stretches of -GKG-, arginine, and lysine residues in the N-terminal region severely overlap. Overall, observation of the N-terminal tail region in J-coupling based SSNMR experiments and solution-state NMR indicates that they are highly dynamic. Chemical shift overlaps of several backbone and side-chain resonances indicate that the H4 N-terminal tail exists as a random coil structure in the NCP.

However, the assignments can be carried over to the hH4 tail domain SSNMR spectra collected with INEPT pulse schemes, since the correlation spectra of the hH4 tail region in 601 ‘Widom’ NCP obtained in solution and the solid-state shown in Figure 24 overlay very well. Furthermore, these assignments can potentially be utilized for studies of histones in the different chemical environments- nucleosome arrays (Chapter 5), chromatin fibers, nucleosomes with natural DNA sequence (Chapter 5), and for protein binding studies.

Figure 24. Correlation spectra of histone H4 N-terminal tails in 601 NCP. Left: ^{15}N - ^1H (upper panel) and ^{13}C - ^1H (lower panel) correlation spectra of the tail domain of ^{13}C , ^{15}N -labeled histone H4 in 601 NCP. Right: Overlay spectra of solution-state NMR (blue) with the SSNMR (red) spectra obtained using HSQC and INEPT pulse schemes respectively.

Results and Discussion: Structure and Dynamics of Histone H3 in the NCP

4.1. HISTONE H3

Histone H3 forms a tetramer with H4 interacting with the phosphates of the DNA minor grooves at the entry and exit points. The histone-histone interface formed by the two H3 histones in the H3/H4 tetramer is proximal to the dyad (Figure 2D). Several amino acids in the globular domain of H3 are positioned such that they are accessible on the lateral surface of the nucleosome. H3 possesses the longest N-terminal tail among other core histones, that project through the nucleosomal DNA and are also accessible on the surface (Luger, Mader et al. 1997). H3 residues buried in the core and on the lateral surface of the nucleosome effectuate crucial functions, particularly transcription, DNA repair, and regulation of chromatin structure (Mersfelder and Parthun 2006, Lawrence, Daujat et al. 2016).

In comparison to other histones, tens of PTMs have been discovered on histone H3 (~30 identified so far) and are crucial for various functions. For example, H3K56 acetylation is shown to effectuate the DNA damage response (Masumoto et al. 2005) and the regulation of DNA transcription (Xu et al. 2005). H3K79 methylation is essential for the maintenance of heterochromatin (Jones et al. 2008). In addition, PTMs on one of the histones can influence the PTM on H3. For example, trimethylation of H3K79me3 and H3K4me3 co-localizing on active chromatin is dependent on the monoubiquitination of H2B (Nakanishi, Lee et al. 2009). Various PTMs on both H3 and CENP-A (a variant histone of H3 in centromeres) nucleosomes contributes to the formation and regulation of centromere-specific chromatin (centrochromatin) and assembly of kinetochore during cell division (Fukagawa 2017).

In this chapter, the structure and dynamic characterization of histone H3 in the NCP by SSNMR is discussed. The characterization strategy is the same as the NMR study of histone H4 in the 601 ‘Widom NCP’ (3.3. SSNMR studies of histone h4 globular domain in the NCP). Sequential SSNMR resonance assignment was carried out on the globular domain and N-

terminal tails of H3 in the human nucleosome reconstituted with 601 ‘Widom’ DNA of 145 bp (Lowary and Widom 1998). NMR chemical shift-based secondary structure was derived, followed by experiments to measure the site-specific dynamics.

4.2. SAMPLE QUALITY

Human histones including the ^{13}C , ^{15}N -labeled hH3, and 601-DNA purified using the method described in the previous sections (2.1. Expression and purification of human histones) was analyzed to be pure (Figure 52 and Figure 54). Histones were refolded into HO (Figure 53) and reconstituted into NCP together with the 601-DNA. The optimum HO:DNA molar ratio used for reconstitution was 0.87, which was determined by a small-scale titration (HO:DNA molar ratio ranging from 0.85–1.05) at a constant DNA concentration of 0.5 mg/ml (Figure 25). The quality of 601-NCP containing the ^{13}C , ^{15}N -labeled hH3 was outstanding as analyzed for both DNA and histones on 6% native PAGE (left panel in Figure 26) and 18% SDS-PAGE (right panel in Figure 26) respectively. About 350 mg/ml NCP was sedimented into the SSNMR rotor, the concentration of which falls in the *in-vivo* heterochromatin concentration (Bohrmann, Haider et al. 1993, Weidemann, Wachsmuth et al. 2003, Bertin, Mangenot et al. 2007). The sample that was gel-like precipitate has about 65% hydration as determined by ^1H NMR spectrum.

Figure 25. Reconstitution of 601 'Widom' NCP with ^{13}C , ^{15}N - labeled H3 analyzed on 6% native PAGE. Small-scale titration of HO:DNA molar ratios ranging from 0.85-1.05.

Figure 26. Reconstitution of 601 'Widom' NCP with ^{13}C , ^{15}N -H3 labeled. Left: NCP analyzed on 6% PAGE. Right: Individual histones (including ^{13}C , ^{15}N -H3) in histone octamer assessed on 18% SDS-PAGE.

4.3 SSNMR STUDY OF THE H3 GLOBULAR DOMAIN IN THE NCP

4.3.1 Chemical shift assignment

Figure 27. 1D ^1H - ^{13}C CP SSNMR spectrum of histone H3 in the Mg^{2+} sedimented 601 ‘Widom’ NCP with chemical shift ranges of protein carbons.

All SSNMR experiments were collected using CP of less abundant $\frac{1}{2}$ spin nuclei (^{13}C , ^{15}N) at a sample temperature of $\sim 12^\circ$. The quality of hH3 in the Mg^{2+} sedimented NCP is outstanding, which is indicated by the characteristic carbon chemical shifts of a folded protein as shown in the 1D ^{13}C spectrum in Figure 27.

The 2D and 3D SSNMR experiments including - DARR, NCA, NCO, NCACX, NCOCX, and CANCO were conducted to facilitate sequential resonance assignments for the hH3 in the 601-NCP. ^{13}C , ^{15}N -labeled hH3 constitutes 16% of NCP by weight. The well dispersed and outstanding resolution of ^{13}C , ^{15}N -labeled hH3 DARR spectrum indicates that the NCP sample is homogenous (Figure 30). The globular domain of hH3 constituting residues R42–G132 was unambiguously assigned by sequential resonance assignments using the 2D and 3D experiments (representative sequential walk for residues are shown in Figure 28), with few exceptions (F78–D81) as illustrated in Figure 29. The absence or weak intensities of F78–D81 resonances indicate that they are relatively flexible in comparison to the rest of the

globular domain. The unambiguous chemical shift assignment of the hH3 globular domain backbone resonances will facilitate NMR studies of the structure and internal mobility of hH3 in nucleosome arrays, chromatin fibers, and NCP constructed with natural sequences.

Due to decreased dipolar couplings in the N-terminal tail region of hH3 (A1-Y41), they were not detectable in dipolar-coupling based experiments. We used J-coupling based experiments to characterize them in the sample (4.4. SSNMR study of h3 N-terminal tail in the NCP). The complete chemical shift assignments are deposited on the BioMagResBank BMRB: www.bmrb.wisc.edu) (Ulrich, Akutsu et al. 2008, Romero, Kobayashi et al. 2020) site under accession number 28035. Overall, observation of residues R42-G132 in the dipolar-coupling based experiments and the absence of A1-Y41 indicates that the globular domain is well ordered and rigid, and the N-terminal tails remain highly flexible in agreement with previous studies (Gao, Nadaud et al. 2013, Xiang, le Paige et al. 2018).

Figure 28. SSNMR based sequential resonance assignment of residues T107-A111 of hH3 in the 601-NCP. Shown here are the ^{15}N strip plots extracted from 3D NCACX (red), CANCO (black), and NCOCX (blue) spectra used in the sequence-specific resonance assignment of residues T107 to A111.

The black dash line indicates the connections made from $N_{(i)}$ to $C'_{(i-1)}$ to $Ca_{(i-1)}$ in the ^{15}N dimension of amino acid i . Adapted from (Shi, Prasanna et al. 2020).

Figure 29. Sequential assignment graph showing carbon and nitrogen spins of hH3 R42–R132. The assigned and unassigned spins are shown in black and grey circles, respectively.

Figure 30. 2D ^{13}C - ^{13}C DARR spectrum of hH3 in the Mg^{2+} sedimented 601 'Widom' NCP with representative resonance assignments. Data was collected with 100 ms DARR mixing time.

4.3.2. Secondary structure

We conducted secondary structure prediction using the obtained chemical shifts with TALOS-N (Shen, Delaglio et al. 2009). The comparison of TALOS-N based secondary structure of the globular domain of hH4 in the Mg^{2+} sedimented NCP concurs well with the secondary structure derived from X-ray crystallography (PDB ID- 3LZ0) as illustrated in

Figure 31. The hH3 in the sedimented NCP show all the characteristic features of four α -helices α N, α 1, α 2, and α 3 linked by LN, L1, and L2 loops. Overall, these structural analyses indicate that the majority of the hH3 form continuous helices constituted by G45–Q55, I63–Q76, Q85–I112, and P121–I130 which are connected by the loop regions. Two short stretches of amino acids L82–F84 and R116–T118 form the β -strands.

Figure 31. Illustration of comparison of NMR based secondary structure prediction of hH3 in the 601 ‘Widom’ NCP with X-ray crystallography structure PDB ID:3LZ0. (A) Terminal residues 1–42 and 132–135 are not shown. (B) Secondary TALOS-N structure prediction for hH3 in NCP. Asterisk marks K79, of which the secondary structure is not derived from the chemical shifts due to a lack of resonance assignments. (C) Schematic secondary structure representation of H3 in the X-ray structure of NCP (PDB: 3LZ0). The cylinders represent α -helix and the arrows represent β -sheet. Reprinted from (Shi, Prasanna et al. 2020).

4.3.3. Dynamics in the ns- μ s timescale

The site-specific dynamics for hH3 in the NCP were elucidated using the 3D-DIPSHIFT (Munowitz, Griffin et al. 1981) experiment with the implementation of R12₁⁴ symmetry sequence (Levitt 2002). 46 isolated peaks were observed for hH3 in the NCP in the 2D NCA spectrum. The ¹H-¹⁵N and ¹H-¹³C α dipolar line shapes were extracted unambiguously from the indirect recoupling dimension identifying the sites from the NCA correlations. These were fitted to obtain the dynamically averaged dipolar coupling values. Experimental dipolar line shape fits are shown in Figure 32 and Figure 33. The order parameters, S_{NH} and $S_{(\text{CH})\alpha}$ obtained (ratio of experimental dipolar coupling constants to values in the rigid limit) for ¹H-¹⁵N and ¹H-¹³C α are summarized for individual residues in Figure 34 A and B. The HC and HN dipolar coupling constants obtained are indicated in Table 16. Overall, the absence of large-amplitude motions in the ns- μ s timescale across the globular domain of hH3 in the NCP signifies that the amino acids are compactly packed and well ordered.

Amino acids in the loops LN and L1 exhibit slightly smaller order parameters compared to the rest of the hH3 globular domain in the μ s-ns timescale (Blue dashed line in Figure 34A and B). These loops are relatively loosely packed in the core, exhibiting higher flexibility (highlighted in Figure 34 C). Contrarily, loop L2 exhibits slightly larger order parameters comparable to those of the helix regions, possibly due to the interaction through hydrogen bonds and salt bridges with the DNA in the dyad region

Figure 32. Site-resolved experimental (dotted) and simulated (solid) ^1H - ^{15}N dipolar line shapes for hH4 protein in the NCP. The experimental line shapes are extracted from a 3D DIPSHIFT with R12,⁴ symmetry dipolar recoupling pulses Reprinted from (Shi, Prasanna et al. 2020).

Figure 33. Site-resolved experimental (dotted) and simulated (solid) ^1H - $^{13}\text{C}\alpha$ dipolar line shapes for hH4 protein in the NCP. The experimental line shapes are extracted from a 3D DIPSHIFT with R12_1^4 symmetry dipolar recoupling pulses Reprinted from (Shi, Prasanna et al. 2020).

Figure 34. Dipolar order parameters (A) S_{NH} and (B) S_{CH3} for hH3 in the Widom ‘601’ NCP. The red box marks F78-D81 absent in the NCA spectrum. (C) Regions of hH3 exhibiting relatively smaller dipolar order parameters are highlighted in red (PDB: 3LZ0) (Vasudevan, Chua et al. 2010). hH3 residues are colored blue and the rest of the NCP is colored grey Adapted from (Shi, Prasanna et al. 2020).

4.3.4. Dynamics in the μ s-ms timescale

Normalized cross peak intensities in the 3D CANCO spectrum (described in page 41), were used in interpreting the site-specific motions in the μ s-ms timescale for hH3 globular domain in the 601-NCP (similar to hH4 dynamics in the 601-NCP described in 3.3.4. Dynamics in the μ s-ms timescale). About 76 site-resolved cross peak intensities of the globular domain

of hH3 in the NCP is resolved in Figure 35A and mapped on to the X-ray structure PDB ID-3LZ0 (Vasudevan, Chua et al. 2010) in Figure 35 B.

Four stretches of amino acids display normalized CANCO cross-peak intensities smaller than 0.5, suggesting pronounced motions in the μ s–ms timescales in these regions, P43-R52 in the α N helix, I62-F67 in LN and α 1 helix, and L1 (highlighted in Figure 35B). Relatively larger-scale ms- μ s dynamics are observed for P43-R52 in α N helix. A previous study found altered nucleosome sliding and unwrapping in H3 with site-mutation in the α 1 and N-terminal tail (Ferreira, Somers et al. 2007). The hH3 α N is located near the DNA entry-exit and is sandwiched between DNA, hH4 L1, and the hH2A C-terminus. A mutagenesis study suggested that the hH2A C-terminal loop participates in regulating nucleosome mobility (Flaus, Rencurel et al. 2004).

In addition, we observed higher flexibility for the hH4 L1 (described in 3.3.4. Dynamics in the μ s-ms timescale). Consequently, these dynamic regions overlap with the histone residues linked to the functional response to modifications. L1 is the most dynamic region in the ms- μ s timescale. In particular, the absence of CANCO correlations demonstrates the extremely high mobility of F78-D81 that resides at the DNA-free surface of the NCP and does not interact with DNA. Recent studies showed that this stretch is involved in gene regulation and harbors several novel PTMs including acetylation/methylation of K79 (Garcia, Hake et al. 2007) and phosphorylation of T80 (North, Javaid et al. 2011). The fact that this region does not interface directly with DNA leads to the possibility of PTM based signaling by modulation of this region together with the internal dynamic networks observed in both H3 and H4 (Shi, Prasanna et al. 2020).

Overall, we have shown the internal motions exist in the globular domain of hH3 in 601 NCP in the μ s-ms timescales.

Figure 35. (A) CANCO peak intensities of hH3 in the Widom ‘601’ NCP. The Redline in box 3 indicates F78-D81 which is absent in the spectrum. (B) hH3 residues in regions 1, 2, and 3 from A, exhibiting relatively low peak intensities are highlighted in red on X-ray structure PDB ID-3LZ0 (Vasudevan, Chua et al. 2010). hH3 residues are colored blue and the rest of the NCP is colored grey. Adapted from (Shi, Prasanna et al. 2020).

4.4. SSNMR STUDY OF H3 N-TERMINAL TAIL IN THE NCP

We obtained 2D ^{13}C - ^1H and ^{15}N - ^1H correlation spectra using the INEPT pulse scheme that is based on J-coupling. The N-terminal tail domains of hH3 in the NCP are highly dynamic and are thus observable in these experiments (upper and lower left panel of Figure 36). The resonances obtained agree well with the previous SSNMR study of H3 tails in 17 mer nucleosome array (Gao, Nadaud et al. 2013) and solution NMR study of ^{15}N -H3 tails in the NCP (Morrison, Bowerman et al. 2018).

Using the same method described in 3.4. SSNMR studies of the histone H4 tail region in the NCP, we carried out sequential resonance assignments of the hH3 tail region. About 80% of the residues in the N-terminal tail region of hH3 in the NCP A1-Y41 were assigned and illustrated for several residues in the 2D ^1H - ^{15}N HSQC and ^1H - ^{13}C HSQC spectra (upper and lower panel of Figure 36). The majority of the backbone amide resonances are well dispersed as evidenced in the ^1H , ^{15}N -HSQC spectrum (upper left panel on Figure 36) complying with the previous solution NMR study of ^{15}N -H3 tails in the NCP (Morrison, Bowerman et al. 2018). Backbone and side-chain resonances of residues lysine, threonine, arginine, alanine, and glutamine overlap due to the disordered structural features.

These assignments can be carried over to hH3 tail domain SSNMR spectra collected with INEPT pulse schemes since the correlation spectra of the hH3 tail region in 601-NCP obtained in solution and solid overlap (right panels of Figure 36). Overall, observation of the N-terminal tail region in J-coupling based SSNMR experiments and solution NMR indicates that the N-terminal tails stay highly dynamic in the precipitated NCP. hH3 N-terminal tail exists as a random coil structure in the NCP manifested by the chemical shift overlap of several backbone and side-chain resonances. Furthermore, these assignments facilitate the NMR study of hH3 in different environments- nucleosome arrays, chromatin fibers, NCPs with various DNA sequences, and protein binding studies.

Figure 36. Correlation spectra of the tail region of histone H3 in the 601 ‘Widom’ 145bp NCP. Left: ^{15}N - ^1H (upper panel) and ^{13}C - ^1H (lower panel) correlation spectra obtained using solution NMR. Right: Overlay spectra of solution-state NMR (blue) with the SSNMR (red) spectra obtained using HSQC and INEPT pulse schemes, respectively.

**Results and Discussion:
Structure and Dynamics of
Histones in the Nucleosome
Array and Telomeric NCP**

We used SSNMR spectroscopy to probe the structure and dynamics of histones hH4 and hH3 in nucleosomes constructed with 601 ‘Widom’ DNA of 145bp as described in chapters 3 and 4. Diverse motions that exist in the globular domains of hH4 and hH3 in ns-ms and μ s-ms timescales were also characterized. We then advanced to probe the histone dynamics in the context of nucleosomes in the chromatin fiber (nucleosome array), and, understand the influence of variation in the DNA sequence on the structure and dynamics of the histones in the nucleosomes by SSNMR.

To study the histone structural and dynamic properties of nucleosomes in the chromatin fiber, we chose to use a nucleosome array reconstituted with 12 repeats of 601 ‘Widom’ DNA of 177 bp repeat length (NRL) referred to as a 12-mer array. Nucleosome-nucleosome interactions facilitate the formation of the 10 nm nucleosome fiber (Woodcock and Dimitrov 2001). Residues in histone H4 N-terminal tails (K16-D24) interact with the neighboring acidic patch on the nucleosome (constituting residues E56, E61, E64, D90, E91, E92 in H2A and E102, E110 in H2B), which facilitate the first level of compaction (Kalashnikova, Porter-Goff et al. 2013). The fibers fold into higher-order structures in the hierarchical organization of the long genomic DNA into chromatin (refer to Figure 1). Thus, studying the structure and dynamics properties of histones in nucleosomes with long DNA is key to understand *in vivo* functions. We first conducted SSNMR experiments to study the unique molecular properties of histone hH4 in the 12-mer nucleosome array in comparison with the 601-NCP.

To study the influence of DNA sequence on the histone structure and dynamics, we characterized the NCP reconstituted using DNA comprised of the human telomeric repeat “TTAGGG”. This NCP is made with a telomeric DNA sequence (ATC-(TTAGGG)₂₃TGAT) and is referred to as telomeric NCP (Soman, Liew et al. 2020). Telomeres protect the ends of DNA in eukaryotic chromosomes (Blackburn 1991). Telomeres constitute telomeric DNA,

shelterin complex, telomeric RNA, and nucleosomes. In cells, telomeres exist in highly dynamic states. Throughout the cell cycle, telomeres function to protect the genome by a constant switch between protected and deprotected states (Blackburn 2001). Unprotected telomeres lead to genomic instability (O'Sullivan and Karlseder 2010). The function of telomeres is associated with changes in histone levels impacting the process of molecular aging (Song and Johnson 2018). Telomeres have dual effects in cancer, playing a crucial role in tumor-suppressive mechanism and, in promoting cancer progression (Maciejowski and de Lange 2017).

Characterization of telomeres *in vitro* has been challenging due to its dynamic nature. However, the first high-resolution X-ray crystallography structure of NCP with the human telomeric DNA of TTAGGG repeat has been recently published by our colleagues (Soman, Liew et al. 2020). The important outcome of this study is that telomeric NCP can be crystallized despite the challenges caused by the weak positioning of telomeric DNA to the octameric core. The study suggests that distinct DNA stretching relative to NCPs reconstituted with 601 'Widom' and α -satellite DNA of similar length owing to its weak positioning. We conducted SSNMR experiments to study the effect of this unique property of the histone hH4 and hH3 structure and dynamics in the telomeric NCP.

In this chapter, SSNMR investigations of the ^{13}C , ^{15}N labeled histone hH4 in the 12-mer nucleosome array, and the ^{13}C , ^{15}N labeled hH4 and hH3 in the telomeric NCP are presented and the influence of DNA length and sequence variation on histone structure and dynamics are discussed.

5.3. STRUCTURE AND DYNAMICS OF HISTONE H4 IN THE NUCLEOSOME ARRAY

5.3.1. Nucleosome array sample quality

Human histones including the ^{13}C , ^{15}N -labeled hH4 obtained by the purification method described in an earlier section (2.1. Expression and purification of human histones) was analyzed with 18% SDS PAGE (Figure 52). The quality of the purified DNA used in this study - 12 repeats of 177 bp 601 'Widom' DNA (12-mer DNA) - was evaluated on 0.8% agarose gel (Figure 55). Both 12-mer DNA and histones were pure. Histones were refolded into HO and reconstituted into a 12-mer array by step-wise dialysis. To determine the optimal HO:DNA molar ratio, a small-scale titration was carried out at HO:DNA ratios of 0.9–1.1 and a constant DNA concentration of 0.5 mg/ml. To assess the quality of the nucleosome array, *ScaI* restriction enzyme (NEB) digestion assay was performed, since 601-177 bp repeats are flanked by the *ScaI* restriction site. The digestion products were analyzed using 6% native PAGE and the stoichiometry of histone octamer to 12-mer DNA was determined to be 1:1 (Figure 37). The optimum ratio for the reconstitution of the 12-mer array with ^{13}C , ^{15}N -labeled H4 in the HO, and the 12-601-177 DNA used for large-scale reconstitution was 0.98:1 (to avoid any precipitation owing to pipetting errors on a larger-scale). The quality of ^{13}C , ^{15}N -labeled hH4 and 12-mer DNA in the nucleosome array was excellent as evidenced by the integrity of individual histones on 18% SDS-PAGE (right panel in Figure 38), and the DNA on 0.8% agarose gel (left panel in Figure 38). The sedimented nucleosome array packed into a SSNMR rotor was gel-like precipitate with about 70% hydration as determined by ^1H NMR spectrum. The concentration is about 300 mg/ml which falls within the *in-vivo* heterochromatin concentration (Bohrmann, Haider et al. 1993, Weidemann, Wachsmuth et al. 2003, Bertin, Mangelot et al. 2007).

Figure 37. *ScaI* digestion of nucleosome array (NRL 177 bp) with ^{13}C , ^{15}N -H4 labeled. Digested 12-mer arrays (increasing HO: 177 DNA ratios) analyzed on 6% native PAGE. Small-scale titration of HO: 177bp DNA ratios ranging from 0.9-1.1.

Figure 38. Reconstitution of 12-mer nucleosome array (NRL 177bp) with ^{13}C , ^{15}N -H4 labeled. Left: 12 mer array analyzed on 0.8% Agarose gel. Right: Individual histones (including ^{13}C , ^{15}N -H4) in the 12-mer array assessed on 18% SDS-PAGE.

5.3.2. Structure and dynamics of H4 globular domain

Dipolar-coupling based 2D DARR, NCA, and NCO SSNMR experiments were conducted for the sedimented 12-mer ‘Widom’ nucleosome array of 177bp NRL containing ^{13}C , ^{15}N labeled hH4. The ^{13}C - ^{13}C correlation spectra of the hH4 globular domain in the 12-mer array and 601-NCP overlay remarkably well (Figure 39). This suggests that the hH4 globular domain in the 12-mer nucleosome array possesses the same secondary structure as in the 601-NCP.

Figure 39. 2D ^{13}C - ^{13}C DARR of human histone H4 in 601 NCP (red) and 12-mer array (black). Adapted from (Shi, Prasanna et al. 2018). Copyright Wiley-VCH.

Similar to the approach described in Chapter 3 (3.3.4. Dynamics in the μs -ms timescale), the normalized cross peak intensities of the 2D NCA and NCO spectra were used to discern the site-specific protein motions in the μs -ms timescale. A comparison of the site-resolved cross peak intensities of the hH4 globular domain in the 12-mer 601 ‘Widom’ nucleosome array (NRL-177bp) relative to the 601-NCP is shown in Figure 40.

Deviations in the chemical shift are noticeable for cross-peaks I26–N25 and P32–K31 in the NCO (Figure 40). These residues are adjacent to the hH4 tail residues 16-24 that interact with the acidic patch (formed by residues in H2A and H2B) of the neighbouring nucleosome (Luger, Mader et al. 1997). This interaction mediates higher-order chromatin folding (Dorigo, Schalch et al. 2003). Due to this different chemical environment, the N-terminus may be positioned differently in the nucleosome array leading to chemical shift deviation in SSNMR experiments. This could facilitate future SSNMR studies to probe the structure and dynamics of H2A and H2B in NCPs and nucleosome array to understand the atomic details of inter-nucleosome interaction. These hH4 regions in the nucleosome array exhibit restricted motions compared to the rest of the globular domain. A decrease in F100–G99 NCO cross peak intensity relative to 601-NCP demonstrates the enhanced mobility experienced in the C-terminal region of hH4 globular domain in the nucleosome array. μ s-ms motions are restricted in L1 and α 3 helix regions relative to the rest of the globular domain evidenced by the enhanced peak intensities.

With the few exceptions described above, hH4 globular domain in the nucleosome array exhibits similar mobility as 601-NCP in the μ s-ms timescale as evidenced by the consistent NCA and NCO peak linewidths.

Figure 40. (A) Overlay of the 2D NCA and NCO SSNMR spectra of human histone H4 in the ‘601’ NCP (red) and 12-mer nucleosome array of 177bp NRL (black) with corresponding normalized cross peak intensities. Reprinted from (Shi, Prasanna et al. 2018). Copyright Wiley-VCH.

5.3.3. Structure and dynamics of the H4 tail region

We obtained 2D ^{13}C - ^1H and ^{15}N - ^1H correlation spectra using the J-coupling based INEPT pulse scheme to characterize the hH4 tails in the 12-mer array of 177bp NRL. The characterization approach is described in 3.4. SSNMR studies of the histone H4 tail region in the NCP. The spectra overlay well with the correlation spectra of hH4 in the 601 ‘Widom’ 145bp NCP (both in solution and sedimented NCP), divulging that hH4 tails adopt similar structures in the NCP and the nucleosome array (Figure 41). However, we observe chemical shift deviation for S1 of H4 in the nucleosome array in comparison with that in the NCP, possibly due to different side-chain conformation.

Figure 41. Overlay spectra of the tail region of ^{13}C ^{15}N - labeled histone H4 in the 12-mer nucleosome array with NRL 177 bp (black) with the sedimented 601-NCP using SSNMR (red) and solution-NMR (blue). ^{13}C - ^1H (left panel) and ^{15}N - ^1H (right panel) correlation spectra are obtained using INEPT and HSQC pulse schemes for SSNMR and solution-state NMR, respectively.

5.4. STRUCTURE AND DYNAMICS OF HISTONE H4 AND H3 IN THE TELOMERIC NCP

5.4.1. Sample quality of telomeric NCPs

Human histones including the ^{13}C , ^{15}N -labeled hH3, and hH4 obtained by the purification method described in an earlier section (2.1. Expression and purification of human histones) were analyzed on 18% SDS PAGE (Figure 52) were found pure for the reconstitution studies. Telomeric DNA of 145bp (ATC-(TTAGGG)₂₃TGAT) was purified using the method described in a previous study (Soman, Liew et al. 2020). Qualitative analysis of telomeric DNA was performed using 10% PAGE and 1.5% agarose gel. Histones were refolded into intact HO (Figure 51 and Figure 53) and reconstituted into NCP together with the telomeric DNA. Small-scale titration with HO:DNA molar ratio ranging from 0.85–1.05 and 0.65–0.85 for ^{13}C , ^{15}N -labeled hH4 and hH3 respectively, and a constant DNA concentration (0.5 mg/ml) (Figure 42) was conducted to find the optimum HO:DNA molar ratios- 0.9 and 0.7 for the reconstitution of the telomeric NCP with ^{13}C , ^{15}N -labeled hH4 and hH3 respectively.

The quality of the telomeric NCPs containing either ^{13}C , ^{15}N -labeled hH3 or hH4 was excellent as evidenced by the nucleosomal telomeric DNA on 6% native PAGE (left panel in Figure 43) and the integrity of individual histones on 18% SDS-PAGE (right panel in Figure 43).

Figure 42. Small-scale titration of HO:telomeric DNA of 145bp in molar ratios ranging from 0.85-1.05 analyzed on 6% native PAGE. H4 is uniformly ^{13}C , ^{15}N labeled.

Figure 43. Qualitative analysis of ^{13}C , ^{15}N - labeled H3 and H4 in the telomeric NCP. Left: NCPs analyzed on 6% native PAGE. Right: Integrity of histones in the NCPs analyzed on 18% SDS-PAGE (of which H3 is uniformly ^{13}C , ^{15}N labeled).

5.4.2. Structure and dynamics of histone H4 and H3 globular domains

Dipolar-coupling based 2D DARR, NCA, and NCO SSNMR experiments were conducted for both ^{13}C , ^{15}N labeled hH4, and hH3 in the sedimented human telomeric NCP. The ^{13}C - ^{13}C correlation spectra of telomeric and 601-NCP overlay remarkably well as illustrated in Figure 44 and Figure 45 for hH4 and hH3 globular domains respectively. Overall, hH4 and hH3 globular domains in the telomeric NCP have the same confirmation as in the 601-NCP, congruent with the recently published high-resolution X-ray structure of telomeric NCP (Soman, Liew et al. 2020). However, a few stretches in the telomeric NCP exhibited minor conformational differences, particularly in side-chains as evidenced by the deviations in chemical shifts of A38, V43-S47, K79-T80, and R95-T96 in hH4 (highlighted in Figure 46), and Y54, T80, and I130 in hH3 (highlighted in Figure 47C). Some of these residues interact with the regions of telomeric DNA that exhibit distinct stretching relative to the 601-NCP (Soman, Liew et al. 2020), which are highlighted in Figure 46C.

Similar to the approach described in Chapter 3 (3.3.4. Dynamics in the μs -ms timescale), we used the normalized cross peak intensities in the 2D NCA and NCO spectra to determine the site-specific motions in histones in telomeric NCP in the μs -ms timescale. Comparison of the site-resolved NCA and NCO cross peak intensities of hH4 and hH3 globular domain in the telomeric NCP relative to the 601-NCP are shown in Figure 46A and Figure 47A, respectively (right panels). Strikingly, differences in relative cross-peak intensities are observed across hH4 and hH3. Several cross-peaks are absent in the telomeric NCP as observed in the 2D NCA and NCO spectra (left panel in Figure 46A and left panel in Figure 47A). This observation suggests that several stretches of histone residues in the telomeric NCP exhibiting enhanced mobility in the μs -ms timescale in comparison with 601-NCP, which is highlighted on X-ray structure of telomeric NCP PDB ID: 6KE9 (Soman, Liew et al. 2020) in Figure 46B and Figure 47C.

Figure 44. 2D overlay ^{13}C - ^{13}C DARR (20 ms mixing time) spectra of human histone H4 in 601 NCP (red) and telomeric NCP (black). Reprinted from (Shi, Prasanna et al. 2020).

Figure 45. 2D overlay ^{13}C - ^{13}C DARR (20 ms mixing time) spectra of human histone H3 in 601 NCP (red) and telomeric NCP (black). Reprinted from (Shi, Prasanna et al. 2020).

Figure 46. (A) Overlay of the 2D NCA and NCO SSNMR spectra of human histone H4 in the ‘601’ NCP (red) and telomeric NCP (black) with corresponding normalized cross peak intensities. Peaks that are absent in telomeric NCP are shown with zero intensities with error bars. Asterisks marks peaks that are resolved in Widom ‘601’ NCP, but not in telomeric NCP (due to small chemical shift perturbations and peak broadening). (B) H4 regions that exhibit enhanced us-ms dynamics (zero peak intensities) compared with 601-NCP are highlighted in red on telomeric NCP X-ray structure PDB ID: 6KE9. (C) H4 residues that interact with telomeric DNA in red. Adapted from (Shi, Prasanna et al. 2020)

Figure 47. (A) Overlay of the 2D NCA and NCO SSNMR spectra of human histone H3 in the 601-NCP (red) and telomeric NCP (black) with corresponding normalized cross peak intensities. Red boxes in both graphs highlight residues F78-D81 that are absent in both spectra of 601-NCP and telomeric NCP. Peaks that are absent in telomeric NCP are shown with zero intensities with error bars. Asterisks marks peaks that are resolved in Widom 601-NCP, but not in telomeric NCP (due to small chemical shift perturbations and peak broadening). (B) H3 regions that exhibit enhanced us-ms dynamics (zero peak intensities) compared with 601-NCP are highlighted in red on telomeric NCP X-ray structure PDB ID: 6KE9. (C) H3 residues that interact with telomeric DNA in red. Adapted from (Shi, Prasanna et al. 2020).

5.4.3. Structure and dynamics of H3 and H4 tail regions

Using the methods described in previous chapters (3.4. SSNMR studies of the histone H4 tail region in the NCP), we obtained 2D ^{13}C - ^1H and ^{15}N - ^1H correlation spectra for hH4 and hH3 tails in the telomeric NCP. The spectra (presented in Figure 48 and Figure 49) overlay well with the correlation spectra of hH4 and hH3 in the 601-NCP (both in solution and sedimented NCPs), provide information about similar conformations of hH4 and hH3 tails in 601 and telomeric NCP. S1 backbone and sidechain peak shift was noticeable in the hH4 tail in the telomeric NCP (peaks circled red in Figure 48). This is interesting, as similar peak shifts of hH4 tail in the nucleosome array were observed (peaks circled red in Figure 41). The influence of sequence variation and compaction within the context of the chromatin fiber seems to have a similar effect in this region for hH4 tail in the telomeric NCP and the nucleosome array.

Several new peaks and peak shifts are observed in the ^{13}C - ^1H and ^{15}N - ^1H correlation spectra for the hH3 tails in the telomeric NCP in comparison with the 601-NCP (peaks circled in red in Figure 49), indicating that the dynamic regions of the N-terminal tail in hH3 is further extended. This property of enhanced histone dynamics in comparison with 601-NCP could potentially contribute to crucial functions of telomeres in cells. For example, epigenetic regulation of telomeres, the inheritance of telomere specific PTMs, recruitment of covalent modifiers, and remodelers in response to DNA damage response all require disassembly/assembly of histones and the shelterin complex associated with telomeres (Galati, Micheli et al. 2013).

Figure 48. Overlay spectra of the tail region of ^{13}C ^{15}N -labeled histone H4 in the telomeric NCP (black) and 601-NCP (blue). ^{13}C - ^1H (left panels) and ^{15}N - ^1H (right panel) correlation spectra obtained using INEPT and HSQC pulse schemes for SSNMR (telomeric NCP) and solution-state NMR (601-NCP) respectively.

Figure 49. Overlay spectra of the tail region of ^{13}C ^{15}N - labeled histone H3 in the telomeric NCP (black) and 601-NCP (blue). ^{13}C - ^1H (left panels) and ^{15}N - ^1H (right panel) correlation spectra obtained using INEPT and HSQC pulse schemes for SSNMR (telomeric NCP) and solution-state NMR (601-NCP) respectively.

In summary, histone H4 in the nucleosome array display similar secondary structure and dynamic properties, with restricted mobility in few stretches. Contrarily, the weak positioning of telomeric DNA to histone octamer heightened the relative dynamics across the histones hH4 and hH3 in the telomeric NCP, in comparison to 601-NCP.

Conclusions and Future Perspectives

6.1. CONCLUSIONS

We have demonstrated the potential of SSNMR spectroscopy for structural and dynamic investigations of chromatin by successfully performing multi-dimensional dipolar-coupling based SSNMR experiments for human histones H4 and H3 in the NCP reconstituted with 601'Widom' DNA of 145bp, 12 repeats of 177 NRL nucleosome array, and telomeric (ATC-(TTAGGG)₂₃TGAT) 145 bp NCP. SSNMR spectra obtained are of outstanding resolution and display well-dispersed peaks from the globular domains of hH4 and hH3 in the NCP. We also carried out J-coupling based experiments to characterize the N-terminal tails. Near-complete unambiguous chemical shift assignment was obtained for both globular and tail domains in the 601-NCP which were carried over to probe histones in different environments.

For the very first time, we have demonstrated the usage of SSNMR to probe site-specific motions and amplitudes in μ s–ns and μ s–ms timescales for chromatin. Our studies revealed diverse internal motions in the globular domains of hH4 and hH3 in the 601- NCP. The absence of a large difference in μ s–ns motions across most amino acid residues suggests that globular domains in hH4 and hH3 are structured and rigid. Diverse internal motions in μ s–ms timescales in the globular domains of hH4 and hH3 in the 601-NCP holds potential contributions in understanding the implications of histone dynamics in nucleosome stability, chromatin mobility, and DNA accessibility *in vitro* in the diverse conditions of nucleosome arrays, chromatin fibers, NCPs with variant histones, natural DNA sequences.

Furthermore, we extended the studies to nucleosome in the context of the nucleosome array and to investigate the effects of variations in the DNA sequence on the structure and dynamics of the histones hH3 and hH4 in the nucleosomes. Histone H4 in the nucleosome array exhibited similar secondary structure and dynamic properties, with restricted mobility in few

stretches. Contrarily, the weak positioning of telomeric DNA to the histone octamer heightened the relative dynamics across the histones hH4 and hH3 compared to the 601-DNA.

Our study is the first to have utilized SSNMR spectroscopy to probe the site-specific motions and amplitudes in chromatin at atomic resolution, pioneering a new dimension in the structural studies of chromatin. Extending the study to the chromatosome, longer arrays, perhaps the 30nm fiber (nucleosome array with linker histones), and probing the dynamic properties of chromatin with variant histones, unique DNA sequences, and with PTMs is impending.

6.2. ONGOING WORK AND FUTURE PERSPECTIVES

In the next phase, we intend to carry out SSNMR characterization of various canonical and telomeric DNA templates such as tetrameric repeats with different NRL and SSNMR binding studies of Heterochromatin Protein 1 (HP1) to canonical and telomeric nucleosome arrays. Proteins from Heterochromatin Protein (HP1) family are involved in maintaining a heterochromatin state. HP1 α is one of the isoforms of HP1 in higher eukaryotes (Kumar and Kono 2020). It specifically binds to the trimethylated H3K9, which is one of the hallmarks of heterochromatin and is thought to be crucial in establishing and maintaining facultative heterochromatin. The interaction is rather transient than a stable one as shown by few studies (Fischle et al., 2005; Maison & Almouzni, 2004). HP1 α drives the formation of liquid-liquid phase separation in heterochromatin (Larson et al., 2017). The molecular mechanism that controls the dynamic interaction of HP1 and H3K9 has not been thoroughly established. Having a well-established method to use SSNMR spectroscopy on chromatin, we intend to examine this interaction. Preliminary solution-NMR investigations on HP1 α binding to hH3 in

the 601-NCP is ongoing, with subsequent studies of this interaction in telomeric NCP and telomeric array.

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Appendix

Chemical	Manufacturer
40 % Acrylamide solution	Bio-Rad
Ammonium persulfate, APS	APS Bio-Rad
Ampicillin sodium salt	USB
2 % Bis-acrylamide solution	Bio-Rad
β -mercaptoethanol	Sigma
Chloramphenicol	Sigma
Chloroform	Fisher Scientific
Coomassie Brilliant Blue R-250	Bio-Rad
Dimethyl sulfoxide, DMSO	Fisher Scientific
Dithiothreitol, DTT	Gold BioTechnology
Ethanol	Merck
Ethylenediaminetetraacetic acid, EDTA	USB
Glacial acetic acid	Merck
Glucose	Sigma
Glycerol	USB
Glycine	Bio-Rad
Guanidinium hydrochloride	Sigma
HEPES	Sigma
Imidazole	Sigma
Isoamyl alcohol/ isopentyl alcohol/ 3-methyl-1-butanol	Sigma
Isopropanol/ 2-propanol/ isopropyl alcohol	Merck
Isopropyl-thiogalactopyranoside, IPTG	Gold BioTechnology
Magnesium chloride hexahydrate	USB
4-Morpholinoethanesulfonic acid, MES monohydrate	Fluka
Phenylmethanesulfonyl fluoride, PMSF	Fluka

Phenol solution	Sigma
Polyethylene glycol, PEG-6,000	Sigma
Potassium acetate	Sigma
Potassium dihydrogen phosphate, KH₂PO₄	Merck
Potassium chloride	USB
Potassium hydrogen phosphate/ di-potassium hydrogen phosphate, K₂HPO₄	Merck
Sodium acetate	Sigma
Sodium chloride	Merck
Sodium dodecyl sulfate, SDS	Bio-Rad
Sodium hydroxide	Merck
Tetramethylethylenediamine, TEMED	Bio-Rad
Tris-base	Vivantis
Triton X-100	Bio-Rad
Trizma base	Sigma
Urea	USB
Magnesium chloride hexahydrate	USB
4-Morpholinoethanesulfonic acid, MES monohydrate	Fluka
D₂O	Sigma

Table 7. Chemicals used and the respective manufacturers.

Buffer	Composition
Alk. Lysis buffer I	50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl pH 8.0
Alk. Lysis buffer II	200 mM sodium hydroxide, 1% SDS
Alk. Lysis buffer III	4 M potassium acetate, 2 M acetic acid
TEK 1.4	10 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 1.4 M KCl
TEK 1.2	10 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 1.2 M KCl
TEK 1.0	10 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 1.0 M KCl
TEK 0.8	10 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 0.8 M KCl
TEK 0.6	10 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 0.6 M KCl
TEK 0.01	10 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 0.01 M KCl

Buffer A (telomeric DNA ion-exchange)	20 mM Tris (pH 7.5), 1 mM EDTA, 300 mM LiCl
Buffer B (telomeric DNA ion-exchange)	20 mM Tris (pH 7.5), 1mM EDTA, 750 mM LiCl
Wash buffer	50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1mM 2-mercaptoethanol
Wash buffer with 1% Triton X-100	50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1mM 2-mercaptoethanol, 1% Triton X-100
SAUDE-1000	7 M Urea, 20 mM sodium acetate, 1 M NaCl, 5 mM 2-mercaptoethanol, 1 mM EDTA
SAUDE-200	7 M Urea, 20 mM sodium acetate, 200 mM NaCl, 5 mM 2-mercaptoethanol, 1 mM EDTA
S-200 unfolding buffer	7 M Gdn-HCl, 20 mM sodium acetate (pH 5.2), 10 mM DTT
TE (10, 50)	10 mM Tris-HCl pH 8.0, 50 mM EDTA
TE (10, 0.1)	10 mM Tris-HCl pH 8.0, 0.1 mM EDTA
HO unfolding buffer	7 M Gdn-HCl, 10 mM Tris-HCl (pH7.5), 10 mM DTT
Refolding buffer (RB high)	2 M NaCl, 20 mM Tris-HCl (pH7.5), 1 mM EDTA, 5 mM 2-mecaptoethanol
Refolding buffer (RB low)	20 mM Tris-HCl (pH7.5), 1 mM EDTA, 5 mM 2-mecaptoethanol
TAE (50×)	Tris base 242 g, 57.1 mL acetic acid, 100 mL EDTA (0.5 M), and water up to one litter. pH 8.0
TBE (10×)	Tris base 108 g, Boric acid 55 g, 40 mL EDTA (0.5 M), water up to one litter. pH 8.0
TB (10×)	Tris base 108 g, Boric acid 55 g, and water up to one litter. pH 8.0
SDS-PAGE running buffer (10x)	250 mM Tris-base, 1.92 M glycine, 1 % SDS
Trace metal mix/litre	5 g Na ₂ EDTA to 800ml water and pH set to 7.5, FeCl ₃ (.6H ₂ O) 0.5 g (0.83 g), ZnCl ₂ 0.05 g, CuCl ₂ 0.01 g, CoCl ₂ .6H ₂ O 0.01 g, H ₃ BO ₃ 0.01 g, MnCl ₂ .6H ₂ O (.4H ₂ O) 1.6 g (1.35 g), filter sterilized
NMR Buffer	20mM Sodium phosphate buffer pH 7.5, 100mM NaCl, 1mM EDTA, 1mM DTT, 0.04% Sodium Azide, 50uM DSS, 8% D2O

Table 8. Buffers used and their compositions.

Bacterial medium	Composition/1L
2x TY	16 g Bacto Tryptone, 10 g Bacto Yeast extract, 5 g NaCl, 0.1 % glucose,
Terrific Broth (TB)	12 g Bacto Tryptone, 24 g Bacto Yeast extract, 17 mM KH ₂ PO ₄ , 72 mM K ₂ HPO ₄ (1 M), 0.4 % glycerol
M9 salt (1X)	Na ₂ HPO ₄ •7H ₂ O- 12.8 g, KH ₂ PO ₄ - 3 g, NaCl- 0.5g, NH ₄ Cl- 1 g
M9 minimal media	M9 salts- 1X, 1M MgSO ₄ , 1M CaCl ₂ , 13C Glucose (from CIL) - 0.2 % (w/v), BME vitamins (100X from Sigma) – 0.25X, Trace metal mix (1000X)- 0.25X

Table 9. Growth media used and their compositions

Enzyme	Manufacturer
<i>EcoRV, ScaI-HF</i>	New England Biolabs, Inc.
RNase A and Proteinase K	Thermo Fisher Scientific.

Table 10. Enzymes used and the manufacturers

Ladder	Supplier
PageRuler Plus Prestained protein ladder	Thermo Scientific
Gene Ruler 1 Kb DNA ladder	Thermo Scientific
Gene Ruler 100 bp DNA ladder	Thermo Scientific
Gene Ruler 100 bp plus DNA ladder	Thermo Scientific

Table 11. Molecular markers used in this work.

Composition of 18% Resolving gel			Composition of 5% Stacking gel		
	3ml	6ml		2ml	2ml
1.5M Tris-HCl (pH8.8)	750 µl	1500µl	0.5M Tris-HCl (pH6.8)	500 µl	1000 µl
40% acrylamide	1350 µl	2700 µl	40% acrylamide	250 µl	500 µl
2% bis acrylamide	450 µl	900 µl	2% bis acrylamide	250 µl	500 µl
MQ	387 µl	774µl	MQ	958 µl	1916 µl
10% SDS	30 µl	60 µl	10% SDS	20 µl	40 µl
10% APS	30 µl	60 µl	10% APS	20 µl	40 µl
TEMED	3 µl	6 µl	TEMED	2 µl	4 µl

Table 12. Compositions of SDS-PAGE gels.

Composition of 10% native PAGE		Composition of 6% native PAGE	
	4ml		5ml
5XTBE	1000 µl	5XTBE	250 µl
40% acrylamide	500 µl	40% acrylamide	750 µl
2% bis acrylamide	500 µl	2% bis acrylamide	375 µl
MQ	1916 µl	MQ	3570 µl

10% APS	40 μ l	10% APS	50 μ l
TEMED	4 μ l	TEMED	5 μ l

Table 13. Composition of native PAGE gels.

Composition of 1.5% Agarose gel	0.75g Agarose, 50 ml water
Composition of 0.8% Agarose gel	0.4g Agarose, 50 ml water

Table 14. Composition of Agarose gels.

Residue number	Residue	HC Dipolar Constant	Residue number	Residue	HN Dipolar Constant
28	G28	-19669.291	28	G28	9306.696
30	T30	-19911.9	30	T30	9050.435
31	K31	-19359.131	31	K31	9484.323
33	A33	-19424.278	33	A33	9519.046
36	R36	-19125.422	36	R36	9050.673
37	L37	-19586.485	37	L37	9052.615
38	A38	-19101.06	38	A38	9583.162
39	R39	-17798.637	39	R39	9917.18
40	R40	-18451.124	40	R40	9064.223
41	G41	-20309.231	41	G41	9922.149
42	G42	-19599.491	42	G42	9400.727
43	V43	-18841.28	43	V43	9959.575
44	K44	-19826.638	44	K44	9050
48	G48	-19151.466	48	G48	9050.156
50	I50	-19057.847	50	I50	9674.233
51	Y51	-19561.57	51	Y51	9290.358
54	T54	-20106.137	54	T54	9453.268
55	R55	-17810.75	55	R55	9626.541
56	G56	-18782.797	56	G56	9615.454
57	V57	-16000.003	57	V57	9599.582

59	K59	-19192.6	59	K59	9052.994
61	F61	-19254.048	61	F61	9536.426
63	E63	-19197.613	63	E63	9627.739
64	N64	-19475.91	64	N64	9459.53
65	V65	-20007.51	65	V65	10054.175
66	I66	-20095.028	66	I66	8460
67	R67	-18974.666	67	R67	9578.176
68	D68	-19599.811	68	D68	9415.089
69	A69	-19986.497	69	A69	9434.952
70	V70	-20136.143	70	V70	9552.237
72	Y72	-18204.159	72	Y72	9326.623
75	H75	-19651.824	75	H75	9050.539
76	A76	-20296.978	76	A76	9706.555
77	K77	-19797.01	77	K77	9540.441
78	R78	-18689.846	78	R78	9516.102
79	K79	-19068.893	79	K79	9050.054
84	M84	-19555.194	84	M84	9427.713
85	D85	-19817.632	85	D85	9051.663
86	V86	-19444.989	86	V86	9081.508
87	V87	-19327.372	87	V87	8697.11
88	Y88	-19621.743	88	Y88	9422.336
89	A89	-19067.411	89	A89	9466.844
90	L90	-19821.29	90	L90	9477.256
93	Q93	-19135.015	93	Q93	9046.147
94	G94	-19912.745	94	G94	8461.449
95	R95	-18675.796	95	R95	8930.972
96	T96	-19902.32	96	T96	6913.368
97	L97	-19728.33	97	L97	9323.466

99	G99	-10389.63	99	G99	5781.391
101	G101	-5662.48	101	G101	4694.685

Table 15. Residue-specific HC and HN dipolar constants for hH4 in the 601 NCP

Residue number	Residue	HC Dipolar Constant	Residue number	Residue	HN Dipolar Constant
45	T45	-19669.291	45	T45	9439.026
46	V46	-19911.9	46	V46	9632.451
50	E50	-19359.131	50	E50	9046.637
51	I51	-19424.278	51	I51	9638.942
54	Y54	-19125.422	54	Y54	9049.742
55	Q55	-19586.485	55	Q55	9451.558
56	K56	-19101.06	56	K56	9506.256
57	S57	-17798.637	57	S57	8866.608
59	E59	-18451.124	59	E59	9050.066
61	L61	-20309.231	61	L61	9053.083
63	R63	-19599.491	63	R63	9051.008
66	P66	-18841.28	69	R69	9623.957
69	R69	-19826.638	70	L70	9632.52
70	L70	-19151.466	71	V71	9755.417
71	V71	-19057.847	73	E73	9622.892
73	E73	-19561.57	74	I74	9630.205
74	I74	-20106.137	76	Q76	9051.381
76	Q76	-17810.75	77	D77	9284.1
77	D77	-18782.797	84	F84	8702.628
84	F84	-16000.003	85	Q85	9052.03
85	Q85	-19192.6	87	S87	9053.588
87	S87	-19254.048	88	A88	9353.206
88	A88	-19197.613	89	V89	9616.249

89	V89	-19475.91	90	M90	9642.084
90	M90	-20007.51	91	A91	9347.908
91	A91	-20095.028	92	L92	9643.328
92	L92	-18974.666	93	Q93	9050.588
93	Q93	-19599.811	97	E97	9056.695
97	E97	-19986.497	100	L100	9387.241
100	L100	-20136.143	102	G102	9313.149
102	G102	-18204.159	103	L103	9052.753
103	L103	-19651.824	106	D106	9721.07
106	D106	-20296.978	113	H113	9374.529
113	H113	-19797.01	114	A114	9569.047
114	A114	-18689.846	115	K115	9324.868
115	K115	-19068.893	118	T118	7482.333
118	T118	-19555.194	119	I119	8888.316
119	I119	-19817.632	120	M120	9468.508
120	M120	-19444.989	122	K122	9051.818
121	P121	-19327.372	125	Q125	9483.984
122	K122	-19621.743	128	R128	9050.273
125	Q125	-19067.411	129	R129	9158.165
128	R128	-19821.29	130	I130	9374.615
129	R129	-19135.015			
130	I130	-19912.745			
131	R131	-18675.796			

Table 16. Residue-specific HC and HN dipolar constants for hH3 in the 601 NCP.

Figure 50. Gel filtration purification of ^{13}C , ^{15}N labeled histone H4. Fractions under the H4 peak analyzed on 18% SDS PAGE. M-Protein marker

Figure 51. Gel filtration of ^{13}C , ^{15}N - labeled H4 in histone octamer. Fractions under the HO peak were assessed for the integrity of all four histones on 18% SDS PAGE. M-Protein marker

Figure 52. Gel filtration purification of ^{13}C , ^{15}N -labeled histone H3. Fractions under the H3 peak analyzed on 18% SDS PAGE. M-Protein marker.

Figure 53. Gel filtration of ^{13}C , ^{15}N -labeled H3 in histone octamer. Fractions under the HO peak were assessed for the integrity of all four histones on 18% SDS PAGE. M-Protein marker

Figure 56. Overlay spectra of 1D ^1H - ^{13}C CP (blue) of histone H4 in the Mg^{2+} sedimented 601-NCP with the 1D ^{13}C experiment without CP (red).

Figure 57. 1D ^1H - ^{13}C CP SSNMR spectrum of histone H4 in the Mg^{2+} sedimented 601-NCP. The experiment was collected at 17.851 kHz MAS at various sample temperatures to determine the optimal temperature to achieve the highest S/N. Experiments were collected on a 18.8 T Bruker Avance III HD standard bore spectrometer equipped with a Bruker 3.2 mm H/C/N Efree probe.