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Unspinning chromatin: Revealing the dynamic nucleosome landscape by NMR

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ABSTRACT

NMR is an essential technique for obtaining information at atomic resolution on the structure, motions and interactions of biomolecules. Here, we review the contribution of NMR to our understanding of the fundamental unit of chromatin: the nucleosome. Nucleosomes compact the genome by wrapping the DNA around a protein core, the histone octamer, thereby protecting genomic integrity. Crucially, the imposed barrier also allows strict regulation of gene expression, DNA replication and DNA repair processes through an intricate system of histone and DNA modifications and a wide range of interactions between nucleosomes and chromatin factors. In this review, we describe how NMR has contributed to deciphering the molecular basis of nucleosome function. Starting from pioneering studies in the 1960s using natural abundance NMR studies, we focus on the progress in sample preparation and NMR methodology that has allowed high-resolution studies on the nucleosome and its subunits. We summarize the results and approaches of state-of-the-art NMR studies on nucleosomal DNA, histone complexes, nucleosomes and nucleosomal arrays. These studies highlight the particular strength of NMR in studying nucleosome dynamics and nucleosome-protein interactions. Finally, we look ahead to exciting new possibilities that will be afforded by on-going developments in solution and solid-state NMR. By increasing both the depth and breadth of nucleosome NMR studies, it will be possible to offer a unique perspective on the dynamic landscape of nucleosomes and its interacting proteins.

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1. Introduction

To understand the molecular basis of life requires intimate knowledge of the structures and motions of biomolecules, as well as the interactions between them. NMR has proven to be uniquely and extremely sensitive to these three fundamental aspects of biomolecules and has thus become a powerful tool in structural biology. NMR has made key contributions in diverse areas of structural biology ranging from protein-protein interactions, protein-RNA complexes and protein folding to membrane proteins [1–8]. Focusing on the cell nucleus, NMR has been instrumental in advancing our understanding of protein-DNA interactions that control gene transcription [9]. Here, we focus on the packaging of DNA into chromatin, which represents one of the most fundamental layers of cell biology. Chromatin provides the required structural compaction of DNA to fit in the nucleus and plays crucial roles in controlling cell fate and protecting genome integrity. Chromatin function ultimately depends on the nucleosome, its repeating unit, and thus on the structural and dynamical properties of the nucleosome and its interactions with a wide range of chromatin factors.

The high-resolution structure of the nucleosome was solved by crystallography over 20 years ago [10], offering the first detailed insights into its molecular organization. Ever since, many structures of nucleosomes have been solved, mostly using different histone species, mutants or complexes with small molecules. Essentially the same nucleosome structure has been found in every case. Yet, it is clear that there is a wide and dynamic 'nucleosome landscape': nucleosomes vary in DNA sequence, histone protein composition, as well as epigenetic status, as defined by the covalent modifications attached to DNA and histone proteins. To capture this diversity and sample the complete landscape is a major challenge. When including the many possible nucleosome-protein interactions in this conformational landscape, the challenge is only exacerbated, and this is reflected in the very few structures of nucleosome-protein complexes that have been solved. Moreover, ever since the demonstration that nucleosomes are inherently dynamic assemblies [11] and contain highly disordered histone tails [12], it remains a key question how nucleosome dynamics and the presence of such flexible parts contribute to nucleosome assembly or disassembly, the binding of chromatin factors, and the remodeling of chromatin structure. Finally, nucleosomes are organized in a higher-order chromatin structure, in which they interact with each other and linker histones in a yet unknown and dynamic manner, further expanding the conformational landscape of nucleosomes.

Here, we review the ways in which NMR has met and overcome the challenges in characterizing the structure, dynamics and interactions of nucleosomes. After a brief overview of nucleosome structure and function, we survey the pioneering NMR studies starting in the late sixties of the last century. We then describe the state-of-the-art solution and solid-state NMR (ssNMR) methods that have enabled the high-resolution study of the nucleosome by NMR, and outline the required isotope labeling and sample preparation. The two penultimate sections focus on the application of NMR in studies of the nucleosome and nucleosome-protein interactions. Concluding our review, we will look forward to the exciting opportunities that lie ahead to capture the dynamic nucleosome landscape by NMR.

2. Overview of nucleosome structure and function

Nucleosomes act as the genome's guardian, protecting its integrity by wrapping the DNA around histone octamers and compacting it into higher order chromatin. At the same time, they act as gate keepers by regulating the binding of proteins that carry out DNA-templated processes like transcription, replication and repair. Tight regulation of these fundamental processes is imposed through the structural conformation and modification state of nucleosomes, and is essential for a healthy cell.

The nucleosome core particle (NCP) consists of two copies of histones H2A, H2B, H3 and H4 and accommodates ~147 bp of DNA, wrapped in 1.6 left-handed superhelical turns around the histone octamer (Fig. 1, top left) [10]. This 200 kDa assembly has an approximate disk-like shape with a pseudo-twofold symmetry axis, the dyad, going through the central base pair of the DNA. NCPs that include a varying length of linker DNA are called nucleosomes, whereas binding of the linker histone H1 to the linker DNA forms a chromatosome [13].

The core histones each consist of a long α helix, enclosed by two shorter ones, and a flexible, lysine-rich N-terminal tail (Fig. 1). The histones fold into H2A-H2B and H3-H4 heterodimers forming the so-called 'handshake motif' through extensive hydrophobic interactions between their histone fold domains. H3-H4 dimers form a tetramer and associate with two H2A-H2B dimers to complete the histone octamer. The DNA around it is held in place primarily by histone arginine side chains that penetrate into the DNA minor groove approximately every helical turn, forming three contact points per histone dimer. As extreme bending of the helix is required at these minor grooves, AT base pairs are more easily accommodated there than the more rigid GC base pairs. A sequence with appropriately alternating AT- and GC-rich regions will therefore enforce formation of a nucleosome with a well-defined position. This is the basis of so-called strong-positioning sequences like the Widom 601 sequence [14] that are frequently used for *in vitro* nucleosome reconstitution.

Nucleosomes are the crucial binding platforms for a large variety of proteins and protein complexes that control chromatin biology and define the functional state of chromatin (Fig. 1, bottom).

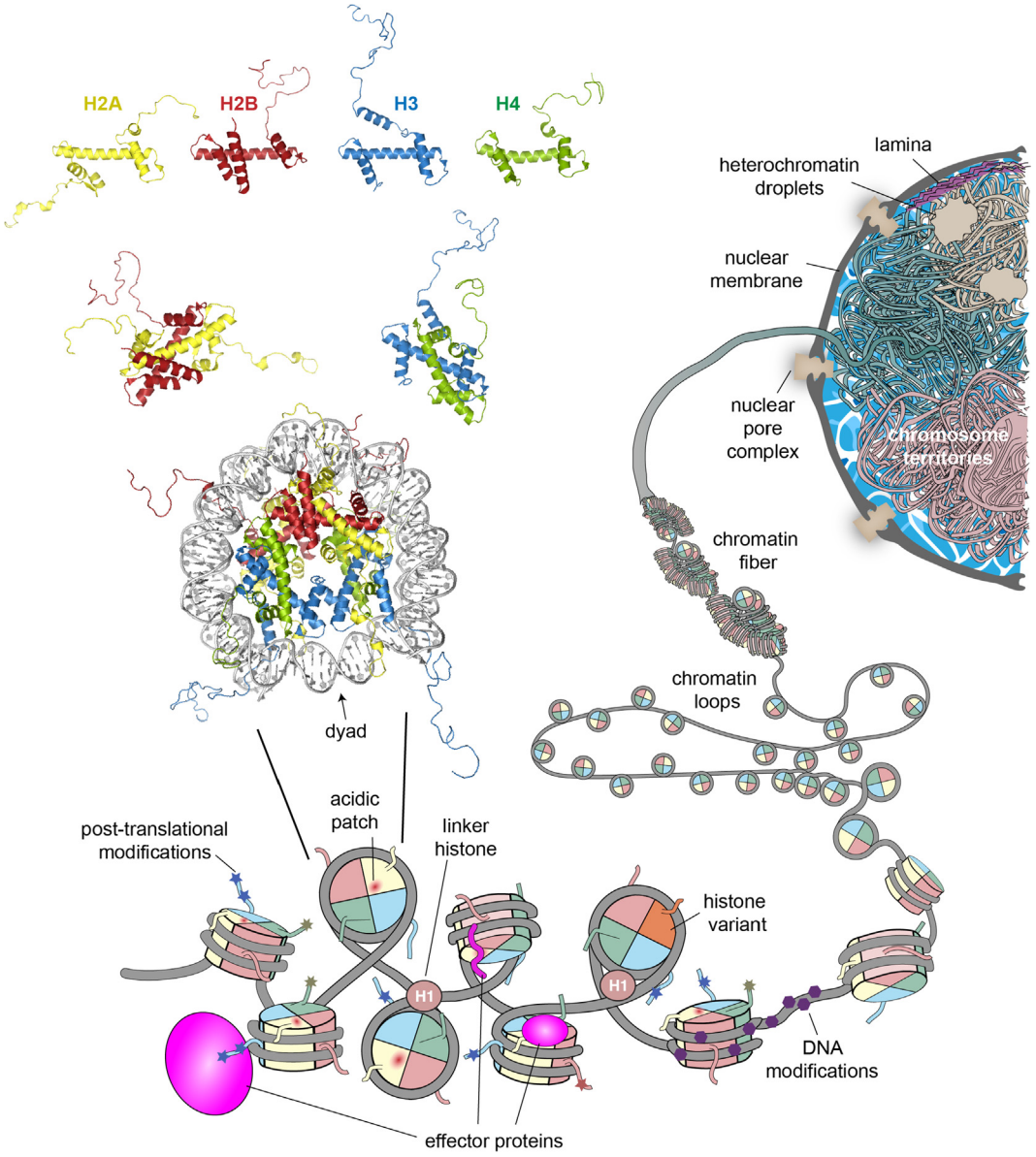

Fig. 1. Hierarchical view of nucleosome and chromatin organization. Histone, histone dimer and nucleosome structures are depicted in cartoon representation in the top left corner, showing histone H3 in blue, H4 in green, H2A in yellow, H2B in red and the DNA in grey. The dyad base pair is indicated by the arrow. The bottom of the figure shows schematically the different modifications and interactions of nucleosomes. The right side of the figure illustrates the compaction into higher order chromatin structures within the cell nucleus.

These include architectural proteins, such as the abundant nucleosomal high mobility group proteins (HMGNs) [15], pioneer transcription factors, i.e. those that are capable of binding target DNA sites that are embedded in a nucleosome [16], and an array of histone modifying enzymes that install or remove post-translational modifications (PTMs) [17,18]. These modifications, which can loosely be referred to as epigenetic marks, can have a direct effect on nucleosome stability or chromatin structure. Many such marks serve to recruit chromatin factors to a distinct genomic location. This recruitment relies on the specific binding of dedicated 'reader' domains to the modification, or a specific combination of modifications. In addition, an important class of proteins can remodel nucleosome and chromatin structure. Chromatin remodeling enzymes convert the energy of ATP to move nucleosomes along the DNA [19,20], while histone chaperones guide the assembly and disassembly of nucleosomes, and the incorporation of histone variants. These non-canonical histones have slightly different properties from their canonical counterparts, changing the structure or stability of the nucleosomes [21,22].

The tremendous breadth of the nucleosome interaction landscape is highlighted by a recent mass spectrometry-based survey that identified hundreds of protein-nucleosome interactions based on *in situ* crosslinking experiments [23]. Nucleosome-binding proteins can bind to the histone tails, the DNA, and also the nucleosome core. Several proteins have been identified that use a single arginine to anchor to the so-called acidic patch, formed by residues from histone H2A and H2B on the core surface of the octamer [24,25] (Fig. 1). Importantly, it is more and more appreciated that many proteins interacting with the nucleosome bind to multiple regions simultaneously, including the nucleosomal DNA, increasing their binding affinity and specificity in the nucleosomal context.

On a larger scale, nucleosomes interacting with each other facilitate the assembly into higher order chromatin structures (Fig. 1,

top right). These structures can include chromatin loops, such as CTCF stabilized loops from an enhancer to a promoter [26], and highly condensed fiber structures [27]. Chromosomes in addition have an internal structure, referred to as topologically associated domains (TADs) consisting of regions within the chromosome that are often in close contact [28]. In addition, phase-separation mechanisms could play a role in chromatin organization, exemplified by the formation of heterochromatin droplets in the presence of phosphorylated heterochromatin protein 1α (HP1α) [29]. The largest structural assemblies within the nucleus are chromosome territories, which have changeable boundaries and are separated by interchromatin compartments [30].

3. Pioneering NMR studies on the nucleosome

Soon after the discovery of histones and nucleosomes as building blocks of chromatin (see Table 1 for an overview of the historic milestones in structural studies of chromatin), NMR was applied to probe their conformational landscape. The earliest studies used histones purified from chicken erythrocytes or calf thymus chromatin by acid and ethanol fractionation [31–33]. Later, it became possible to isolate native histone dimer and tetramer complexes from 'chromatin gel' by protamine replacement of the histones in combination with size exclusion chromatography under non-denaturing conditions [34,35]. Nucleosome core particles could be obtained by micrococcal nuclease (MNase) digestion of isolated chromatin [36]. The samples obtained were obviously inhomogeneous, due to varying DNA sequences, presence of histone variants and PTMs, and possibly associated proteins such as linker histones. As isotope labeling of these materials was not possible, studies were limited to natural abundance NMR experiments, observing ^{1}H, ^{31}P, or ^{13}C nuclei.

3.1. Revealing the nature of histones

Histone proteins were discovered in goose blood cells by Albrecht Kossel in 1884 [37]. They were characterized as a very diverse group of proteins rich in basic residues and likely involved in gene regulation. In 1965, fractionation revealed five histone fractions termed F1, F2A1, F2A2, F2B and F3 [38], which were later renamed histones H1, H4, H2A, H2B and H3, respectively. Histones were isolated under denaturing conditions and refolded by dialysis to water and increasing the salt molarity, which also promotes histone aggregation. Early NMR studies used 1D ^{1}H spectra to deduce the helical segments of individual histones and study their folding and aggregation. The first example is a study by the late Morton Bradbury, who pioneered the application of NMR to study chromatin and whose extensive body of work anticipated much of the research that is still on-going today. This work from 1967 showed that alanine, leucine, isoleucine and valine are part of the folded, helical segment [39]. As the primary sequences of the histones became available [40–43], several studies used this information combined with broadening of aromatic and apolar resonances upon histone folding to conclude that the C-terminal part of the histones is helical and that the N-terminal lysine-rich region remains mobile (Fig. 2A) [44–51]. It was shown that the apolar side of the helical segment facilitates interactions between histones of the same type, while the random coil segment remains mobile in the aggregate [44,45]. Using highly concentrated samples with protein concentrations up to 100 mg/mL, the large chemical shift dispersion of ^{13}C NMR was exploited to show that aggregation does not affect the secondary structure [52–54]. Of note, one report employed a methylation/demethylation cycle to obtain ^{13}C-labeled methionine methyl groups to study the self-aggregation of H2B, a first attempt in isotope-labeling [55].

After the milestone discoveries by Thomas and Kornberg that histones associate pairwise, H3 to H4 and H2A to H2B, and that these pairwise interactions occur in the repeating unit of chromatin [56,57], the attention shifted from self-aggregation of histones to their native interactions and tertiary structure. In 1976, Moss et al. [58,59] isolated native H3-H4 and H2A-H2B complexes from calf thymus and showed by 1D ^{1}H NMR that these have a specific tertiary fold as evidenced by ring-current upfield shifted methyl peaks, and that, while folded, they still contain a large, unstructured region, likely corresponding to the N-terminal region (Fig. 2B). The folding of H3 in refolded histone octamer mixtures was studied by ^{19}F NMR using trifluoro-acetylation of the native cysteines in calf H3 confirming the presence of defined tertiary structures at intermediate ionic strength [60]. To map the interaction surface in native H3-H4 tetramers, mixtures of H3 and H4 fragments were studied by ^{1}H NMR showing that the first ~40 residues of both histones are not required for complex formation [61]. Most lysines and alanines, located in the N-terminal region, were

Table 1
Milestones in structural studies of the nucleosome.

Timeline	Ref.	Key finding	Method
1884	Kossel [37]	Discovery of histone proteins	
1965	Phillips [38]	Five histone fractions	
1969–1972	DeLange, Iwai, Yeoman [40–43]	Histone primary sequences	
1974	Olins [72]	Beads on a string (ν bodies)	EM
1974–1975	Kornberg & Thomas [56,57]	Tetramers and dimers of histones, nucleosome composition	X-ray, cross-linking
1984	Richmond [84]	7 Å structure of the nucleosome	X-ray
1987	Clore [87]	Solution structure of histone H5	NMR
1993	Ramakrishnan [88]	Crystal structure of histone H5	X-ray
1994	Cerf [89]	Solution structure of histone H1	NMR
1997	Luger [10]	2.8 Å structure of the nucleosome	X-ray
1999	Dhalluin [90]	First titration of reader domain with acetyl-Lys histone peptide	NMR
2002	Nielsen, Jacobs [91,92]	First structure of reader domain in complex with methyl-Lys histone peptide	NMR, X-ray
2002	Davey [93]	1.9 Å structure of the nucleosome	X-ray
2005	Schalch [94]	Tetranucleosome structure	X-ray
2006	Barbera [95]	First structure of nucleosome-peptide complex (LANA)	X-ray
2010	Makde [96]	First structure of nucleosome-protein complex (RCC1)	X-ray
2011	Kato [97]	First high-resolution NMR study on the nucleosome and interaction study with (HMGN2), methyl assignments	NMR
2013	Gao [98]	Tail dynamics in nucleosome arrays	ssNMR
2014	Song [99]	30 nm fiber structure	cryo-EM
2016	Moriwaki [100]	H2A-H2B dimer structure	NMR
2018	Xiang, Shi [101,102]	First high-resolution ssNMR study of nucleosome histone core	ssNMR

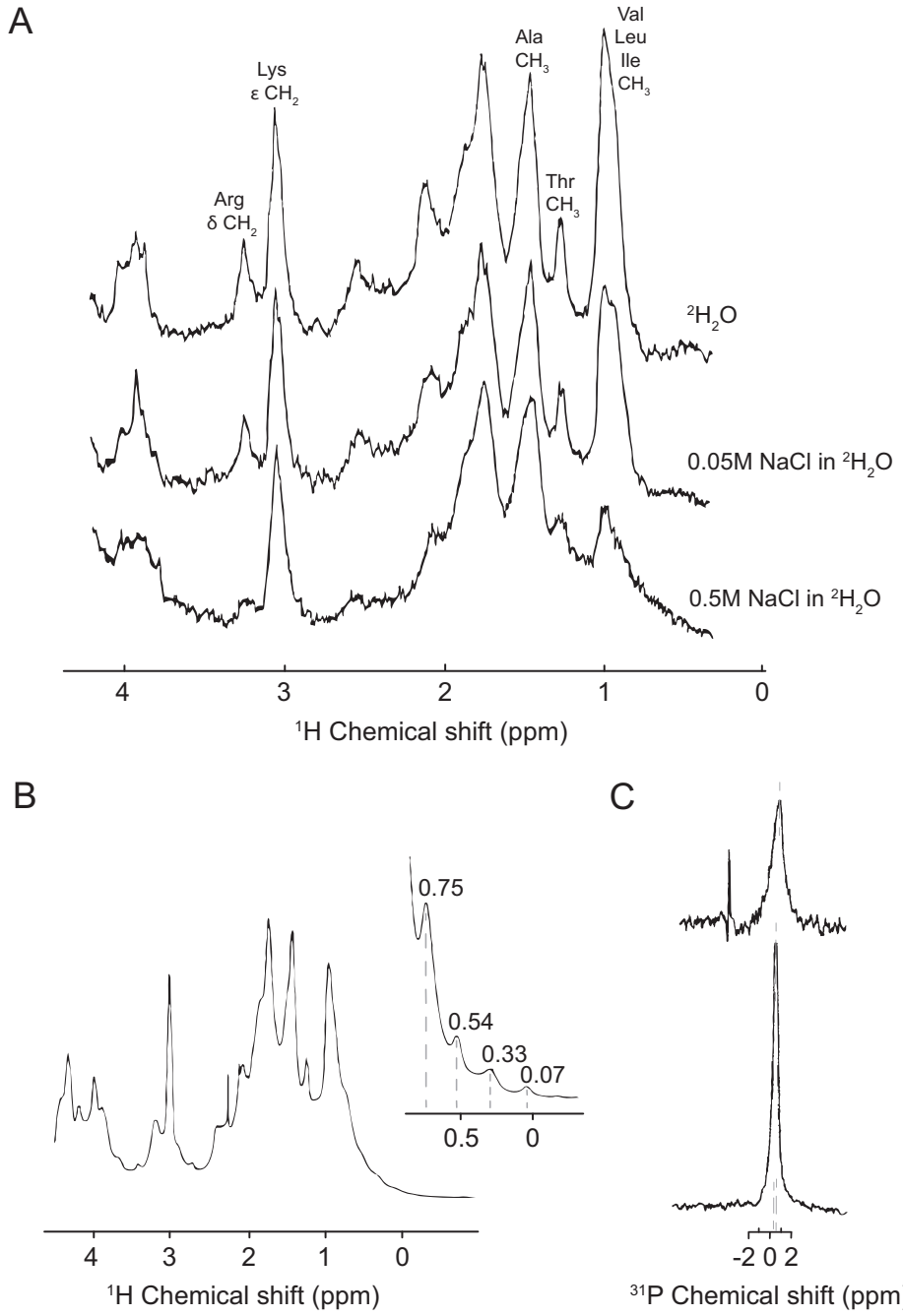

Fig. 2. Pioneering NMR studies on nucleosomes and histones. (A) 1D ^{1}H NMR spectra (220 MHz) of histone H2B showing strongly reduced peak intensity for leucine, isoleucine and valine methyl group resonances at high ionic strength, while the lysine side chain resonance is unaffected. This suggested that leucine, isoleucine and valine experience restricted mobility upon salt-induced folding and interaction while lysines (present mostly in the N-terminal tail) remain mobile. Reproduced with permission from [45]. (B) 1D ^{1}H NMR spectrum (270 MHz) of H2A-H2B dimers with the inset showing a deconvoluted, resolution-enhanced spectral expansion. The ring current shifted apolar resonances suggested that some methyl groups are in close contact with aromatic residues, indicating a specific interaction between H2A and H2B. Reproduced with permission from [58]. (C) 1D ^{31}P NMR spectra (measured at 60 MHz ^{1}H frequency) of nucleosome cores (top) and free nucleosomal DNA (bottom) showing that DNA in the nucleosome has a larger linewidth than free DNA but no significantly different chemical shifts, suggesting that DNA in the nucleosome is not kinked. Reproduced with permission from [71].

shown to remain in random coil state in several different histone complexes [12,62,63]. In the mid 1970s, work from Lilley et al. showed by NMR relaxation measurements that these regions are indeed highly flexible and pushed the notion of 'histone tails' for the first time [12,63].

Next, the role of these tails in the native nucleosome was addressed. Early reports had proposed that the very basic tails would provide the fundamental contacts between histones and the negatively charged DNA [64]. However, a ^{1}H NMR study on the salt-induced dissociation of nucleosomes and tetrasomes argued that only the H3 and H4 tails and not the H2A-H2B tails are bound to the DNA [65]. In addition, trypsinized nucleosomes, lacking the basic tails, were shown to retain a DNA-bound histone core [66]. The Bradbury group showed that H4 tail peptides only weakly bind DNA, and this interaction is abolished upon acetylation, a first example of the study of histone PTMs [67]. Later studies

showed that the histone tails, although not essential for histone-DNA binding and highly mobile even in oligonucleosomes, do interact with the DNA under physiological salt conditions, but much more weakly than do the histone cores [68–70]. The basic tails are suggested to shield neighboring DNA charges in this way, thereby stabilizing higher order chromatin packing.

3.2. 'Unwrapping' the nucleosomal DNA

Acquiring and interpreting NMR spectra of nucleosomal DNA, corresponding to ~100 kDa of the 200 kDa nucleosome assembly, is extremely challenging due to extensive signal overlap and line broadening. Nevertheless, some bold attempts were made in the 1970s to assess the conformational state of DNA in chromatin. As it was known that DNA is present in 'beads' or ν bodies in chromatin connected by linker DNA [72], the DNA inside the beads was assumed to adopt a conformation different from linker DNA, and several models were proposed for the wrapping of DNA around the histone core, either suggesting kinked bending every 10 or 20 bp [73,74], or continuous bending [75,76]. Hanlon et al. [77] were the first to use ^{31}P NMR to probe the DNA conformation in purified, sheared chromatin fragments. They found that the severely broadened ^{31}P signal likely corresponds only to linker DNA between the beads, suggesting a rigid structure and strong interaction of the backbone phosphates with the histones inside the beads. Subsequent studies used MNase-digested chromatin to result in smaller nucleosome particles and thus better spectra. These did not find significant ^{31}P chemical shift perturbations (CSPs) compared to free DNA, suggesting smooth bending rather than kinking (Fig. 2C) [71,78–81]. Scrutiny of upfield-shifted imino ^{1}H resonances suggested local alteration of base pairing near histone-DNA contacts [82,83], in agreement with the first crystal structure of the nucleosome [84]. In 1997, the high-resolution nucleosome structure by Luger et al. [10] definitively confirmed that kinking occurs at several sites in the nucleosomal DNA, but not in the predicted regular way of every 10 or 20 bp.

Following up on the first ^{31}P ssNMR experiments on chromatin by DiVerdi et al. [81], the group of Kyogoku did some pioneering work in the late '80 s on the structure of *in vivo* chromatin by probing the dynamic state of the DNA [85,86]. Using ^{1}H-^{31}P cross-polarization to distinguish the slow-tumbling chromatin from flexible phospholipids and inorganic phosphate, they measured the chemical shift anisotropy of ^{31}P in intact chicken erythrocytes, and concluded that the DNA is highly condensed, supporting the still highly debated presence of a 30 nm fiber.

3.3. Structure of the linker histone

The presence of linker histone H1 was established early on in chromatin research, and it was known to have a high lysine, alanine and proline content and less tendency to aggregate than the core histones. It is the first histone to dissociate from chromatin upon increase of ionic strength and already in the 1960s, it was clear that it can interact with naked DNA. In 1973, a 1D ^{1}H NMR study from Bradbury and colleagues reported histone H1 to be required for the condensation of chromatin and mostly bound to it by its lysine residues [103]. In subsequent NMR papers, the focus has been mainly on identifying the position of the globular segment of this lysine-rich histone fraction [104–111]. The high similarity between NMR spectra of H1 in chromatin and H1 bound to DNA indicated that H1 does not interact with the other histones [106], which was supported by neutron diffraction studies showing that H1 binds to the outside of a nucleosome [112].

Preceded by a preliminary study from the Bradbury lab using solely 1D spectra [113], in the mid 1980s Clore et al. succeeded in obtaining resonance assignments of an avian-specific isoform of the linker histone, histone H5. Using histones purified from chicken erythrocytes, they succesfully applied the homonuclear NOESY-TOCSY approach developed by Wüthrich a few years earlier [114–116]. As one of the first protein structures ever to be determined by NMR, a structural model of H5 was constructed based on 307 interproton distance restraints from NOE data elucidating the helical organization of the protein [87]. Later work in the mid 1990s on the globular domain of the H1 isoform used a recombinant polypeptide and ^{15}N isotope labeling to determine the tertiary structure of the globular domain of linker histone H1 [89,117]. The overall structure was found to be very similar to the H5 crystal structure [88], with small, local structural differences suggested to be responsible for the difference in DNA binding affinity between linker histones H1 and H5.

3.4. Non-histone proteins in chromatin

Apart from histone proteins, chromatin isolated from cells contains a small but significant fraction of non-histone proteins [118]. Particularly abundant are the so-called high-mobility group (HMG) proteins [119]. Proteins of the HMGN subclass bind the nucleosome, and this interaction was the first nucleosome-protein interaction to ever be studied. Again, it was work from Bradbury from the late 70s that showed by 1D ^{1}H NMR that HMGN1 and HMGN2 are in a random coil conformation in their free state, contain a DNA-binding region between residues 15 and 40 and are completely bound to sonicated calf thymus DNA at low ionic strength [120,121]. In 1989, Cook et al. concluded from ^{1}H NMR data that the basic central region of HMGN2 binds strongly to the nucleosome core particle, but they also detected weak binding of the acidic C-terminal region. They assumed that these basic residues would interact with the acidic backbone of the DNA, and the acidic C-terminal region presumably to the core histones [122]. As will be described in Section 6, it took the development of Methyl-TROSY (MeTROSY) NMR to demonstrate that the central basic region binds both the acidic patch on the surface of the H2A-H2B dimer and the DNA simultaneously [97].

3.5. Summary

Well before the development of structure determination by NMR, multidimensional NMR and isotope labeling, NMR had already made an important contribution to the characterization of histone structure and interactions. In the pioneering phase of nucleosome research, before the 1984 structure [84], the composition and the overall shape of the nucleosome was known from X-ray diffraction and electron microscopy [56,57]. During this stage, NMR was able to identify the position of the histone helices and ascertain that H2A-H2B dimers and H3-H4 tetramers have a defined, specific fold. NMR studies were instrumental in establishing the concept of histone tails: a large body of work showed that the N-terminal segments of the core histones are unstructured, highly flexible and weakly associated to the DNA. Finally, NMR was a crucial tool in determining the structure of the linker histones H1 and H5 and in characterizing the interaction of HMGNs with the nucleosome.

4. Modern NMR studies on the nucleosome

The success of modern-day NMR, as with any other structural biology technique, greatly depends on the ability to obtain homogeneous, stable samples, and this is no less true for studies of the nucleosome. Difficulties in reconstituting nucleosomes *in vitro* proved a great challenge for structural studies. The Herculean effort by Luger, Richmond and co-workers to solve the first high

resolution crystal structure of the nucleosome showed that a high affinity DNA sequence was crucial for efficient reconstitution of nucleosomes *in vitro* [10]. Thanks to method development in solution and ssNMR, the challenge of the high molecular weight of the nucleosome was overcome, permitting the atomic-resolution structural and dynamical characterization of nucleosomes. In this section, we will detail the sample production and NMR methods available for such studies.

4.1. Sample preparation

Nucleosomes for NMR experiments are reconstituted from recombinantly expressed histones and nucleosomal DNA of a defined sequence. Histones are expressed separately, purified and refolded into octamers. Subsequent salt gradient-mediated deposition of octamers onto the DNA yields nucleosomes. A great advantage of this stepwise procedure is that it allows differential isotope labeling of the histones to reduce signal overlap.

4.1.1. Production, isotope labeling and modification of histones

Histones are extremely well-conserved proteins, giving the experimenter a certain degree of freedom in the choice of construct. Most NMR studies have used either *Drosophila* (Kay, Bai, Van Ingen labs), *Xenopus* (Narlikar lab) or human histones (Mer, Nishimura, Pervushin & Nordenskiöld labs). Histones are generally overexpressed from codon-optimized plasmids in *E. coli*, according to the protocol published by the Luger lab [123]. As isolated histones are insoluble, they are subsequently purified from inclusion bodies, followed by gel filtration and ion exchange chromatography under denaturing conditions. Yields are typically around 10–50 mg per liter of culture, depending on the histone and culturing medium. Denatured, purified histones can be refolded into histone dimers, tetramers or octamers by dialysis to high salt buffer and purified by size exclusion chromatography with a typical 60–80% final yield. To optimize the yield and prevent aggregation, it is essential to ensure an equimolar ratio of the histones. Because histones carry few aromatic side chains and are devoid of tryptophan residues, determination of the protein concentration is particularly sensitive to contamination with DNA. An additional ion-exchange chromatography step may be required to obtain DNA-free, pure histones [124]. Histones are generally expressed separately but there are a few reports of successful use of H2A-H2B fusion proteins [125,126].

When using uniform $^{15}N/^{13}C$ isotope labeling, production in D_2O-based media with protonated glucose is sufficient to obtain high-quality spectra of histone dimers or the highly flexible tails within the nucleosomes. In addition, uniform labeling has been shown to be suitable for ssNMR studies of the nucleosome (see below) [101,102]. To probe the rigid core of the 200 kDa nucleosome complex by solution NMR, the most suitable labeling strategy is the specific labeling of methyl groups in a highly deuterated background, as introduced by the Kay lab [127]. Specific methyl group labeling is achieved by expressing the histone in fully deuterated M9 medium in the presence of amino acid precursors that are $^{1}H^{13}C$-labeled only at the methyl group of interest. Protocols for labeling the isoleucine-δ1, leucine and valine (ILV), or alanine, methionine and threonine methyl groups are reviewed in Kerfah et al. [128].

Several strategies can be used to incorporate PTMs into histone proteins (reviewed in [129]). However, their combination with isotope labeling remains challenging. Since genetic approaches relying on amber codons typically suffer from low yields, chemical approaches based on modification of an introduced cysteine residue have been used to install a methylated lysine mimic [125,130–132] and a ubiquitinated lysine [125,133]. Histones contain only one conserved native cysteine, H3 C110, which can be substituted to Ala or Ser without loss of function.

4.1.2. Production of nucleosomal DNA

DNA for the reconstitution of nucleosomes can be of varying length and sequence. Most commonly, a strong-positioning sequence is used that ensures efficient reconstitution of nucleosomes. These sequences, such as the non-natural '601' sequence from Widom [14] or the human α satellite repeat [10], are AT-rich in regions where the minor groove faces the octamer to ensure uniform positioning of the nucleosome. Minimum length for nucleosome core particle reconstitution is 145–147 bp; longer sequences will provide linker DNA extending from the core particle.

Nucleosomal DNA can be produced from a multiple-copy plasmid that is amplified in *E. coli* and extracted from the cells by alkaline lysis [123]. The plasmids are subsequently digested to separate the repeats and purified by ion exchange chromatography. This method typically yields about 20 mg of 147 bp 601-DNA from 3 L of culture. Alternatively, nucleosomal DNA can be obtained through large-scale PCR followed by ion exchange chromatography. Although this method is labor-intensive and ideally requires several PCR machines running in parallel, 2–3 mg of 601-DNA can be produced from 4000 50 μL PCR reactions in less time compared to the plasmid method. Plasmid-based production of DNA readily allows isotope labeling by culturing the plasmid in ^{13}C- or ^{15}N-labeled medium, although severe signal overlap due to the poor chemical shift dispersion of DNA can be expected.

4.1.3. Reconstitution of nucleosomes and nucleosome arrays

Nucleosomes are reconstituted by salt-gradient deposition, in which histone octamers and DNA are combined at 2 M salt concentration and gradually dialyzed to low salt conditions to allow the nucleosome to form in a stepwise manner [123]. Also here, accurate determination of octamer and DNA concentration is vital to ensure an equimolar mix and to avoid aggregation. Proper tuning of this ratio will also prevent excess free DNA in the final preparation, which can be determined from native PAGE analysis. Yields for reconstitution are typically around 80–90%. Sample conditions for solution NMR are usually 50–150 μM nucleosomes in a 10–20 mM Tris or NaPi buffer at pH 6–8 and 0–150 mM NaCl or KCl. Experiments are usually recorded at 20–45 °C.

Nucleosome arrays are reconstituted in a similar way by stepwise salt dialysis using multiple repeats of a strong-positioning sequence including linker DNA segments [134]. Octamers and DNA are combined in the presence of lower affinity competitor DNA, to prevent overloading of the DNA with octamers. The arrays are purified by Mg^{2+}-precipitation or by sucrose gradient centrifugation [98]. Increasing the divalent cation concentration in the preparation will promote array compaction and self-association.

4.2. State-of-the-art NMR methods

A wide range of NMR methods is available to study the structural and dynamic properties of (sub)nucleosomal complexes. Depending on the question at hand, a divide-and-conquer strategy may be employed, allowing data to be recorded on histone peptides or histone dimers (~25 kDa) using the entire toolbox of protein solution NMR. In the sections below, we focus on the state-of-art methods that enable the study of intact nucleosomes or nucleosome-protein complexes by NMR, summarized in Fig. 3.

4.2.1. Methyl-based solution NMR

The development of the MeTROSY approach has been instrumental in pushing the molecular size limitations of solution NMR into the MDa range [6,135]. In this approach, side chain methyl

		strategy	structure	dynamics	interactions
histone tail	solution NMR	INEPT-based	NOESY PRE, PCS backbone chemical shifts	^{15}N T_1,T_2, NOE CPMG, CEST	CSPs saturation transfer H/D exchange intermolecular NOESY
	ssNMR	INEPT-based	PRE, PCS backbone chemical shifts		CSPs magnetization transfer
histone core	solution NMR	MeTROSY-based	NOESY, RDC PRE, PCS	Me ^{1}H T_2, S^2 ^{13}C/^{1}H MQ CPMG CEST	CSPs saturation transfer intermolecular NOESY
	ssNMR	CP-based	dipolar recoupling PRE, PCS backbone chemical shifts	dipolar couplings S^2 ^{15}N $T_{1\rho}$	CSPs (1H detection) magnetization transfer

Fig. 3. NMR tool box for the study of nucleosome structure, dynamics and interactions.

groups, typically in isoleucine, leucine and valine, are used as local probes for structure and dynamics. Methyl groups offer three distinct advantages as NMR probes: being at the tip of flexible side chains, they are partially decoupled from the slow overall molecular tumbling, they contain three equivalent protons, and they rotate rapidly around their internal symmetry axis, allowing the exploitation of a line-narrowing effect caused by relaxation interference between different dipolar interactions within the methyl group [136]. The HMQC experiment takes full advantage of this effect and is thus the cornerstone of MeTROSY, offering high-quality, sensitive spectra of large complexes. Importantly, the presence of external protons causes efficient relaxation of the methyl protons, especially for high-molecular-weight systems. Thus, near-complete deuteration of the non-methyl protons is essential to achieve the highest sensitivity. In the context of nucleosome studies, the DNA used for reconstitution is protonated and indeed histone methyl groups close to the DNA are significantly broadened (see also Fig. 4). Likewise, use of protonated binding partners in nucleosome-protein interaction studies will result in peak broadening for interface residues.

In a collaboration between the Kay and Bai labs, the MeTROSY approach was first applied to the nucleosome [97]. The ILV-methyl groups in the nucleosome are reasonably distributed over the tail and core regions, including both the histone octamer surface and interior (Fig. 4A and 4B). MeTROSY spectra of the nucleosome in which one of the histones is ILV-labeled are of excellent quality (Fig. 4C). Assignment of the NMR signals is required for the site-specific interpretation of the structural and dynamical properties they encode. A near-complete assignment (89%) was achieved based on a combination of structure-based NOESY assignment and extensive mutagenesis, and the assigned chemical shifts are reported in the supplementary material of ref. [97]. Using samples in which both methyl groups in Leu and Val residues are ^{1}H/^{13}C-labeled, the corresponding resonances were paired based on strong intra-residual cross-peaks in NOESY spectra recorded with short mixing time. To provide unambiguous check points for the assignment, in total 59 Ile to Leu or Leu/Val to Ile point mutations were made, out of 170 methyl groups in the nucleosome. The assignment was then completed by matching the NOE pattern in a set of six NOESY spectra, obtained on samples containing either ILV-labeled H2B, H3, H4, H2A/H2B, H2A/H3, or H3/H4, to the network of short methyl–methyl distances in the crystal structure. For several Leu/Val residues stereospecific assignments could be obtained.

The workhorse of the MeTROSY suite of experiments is the 2D ^{13}C^{1}H HMQC experiment. High quality 2D correlation maps can typically be obtained in ~1 h on samples containing 100 μM nucleosomes (200 μM of each histone), corresponding to ~5 mg of nucleosomes in a 250 μL Shigemi tube sample. These can not only be used for CSP mapping, but also for the measurement of long-range distance restraints by exploiting either pseudocontact shifts (PCS) or paramagnetic relaxation enhancement (PREs) generated by a paramagnetic spin label [137]. The HMQC pulse scheme is also amenable to implemention of very fast pulsing schemes that enable the study of real-time kinetics [138,139]. In addition, MeTROSY-optimized pulse sequences are available for the measurement of residual dipolar couplings (RDCs) [140], NOE distance restraints, and the study of protein dynamics. Side-chain motions on a fast ps-ns timescale can be extracted from ^{1}H transverse relaxation rates and intra-methyl ^{1}H-^{1}H dipolar cross-correlated relaxation rates [141], while slow μs-ms motions can be studied using ^{13}C^{1}H multiple-quantum Carr-Purcell-Meiboom-Gill (CPMG) relaxation dispersion [142] or methyl-based chemical exchange saturation transfer (CEST) experiments [143,144].

4.2.2. Solid-state NMR

As chromatin is a polymer that can easily be precipitated by the addition of divalent ions, it is a very suitable system to be investigated by ssNMR. Moreover, the breakthrough demonstration that sedimentation of large, soluble protein complexes can result in highly resolved ssNMR spectra [145,146] also opened the door to studies of mononucleosomes or nucleosome core particles by ssNMR.

The few pioneering studies that have applied ssNMR to nucleosomes will be discussed in more detail in the next section. Apart from the lack of intrinsic size limit, ssNMR offers three additional

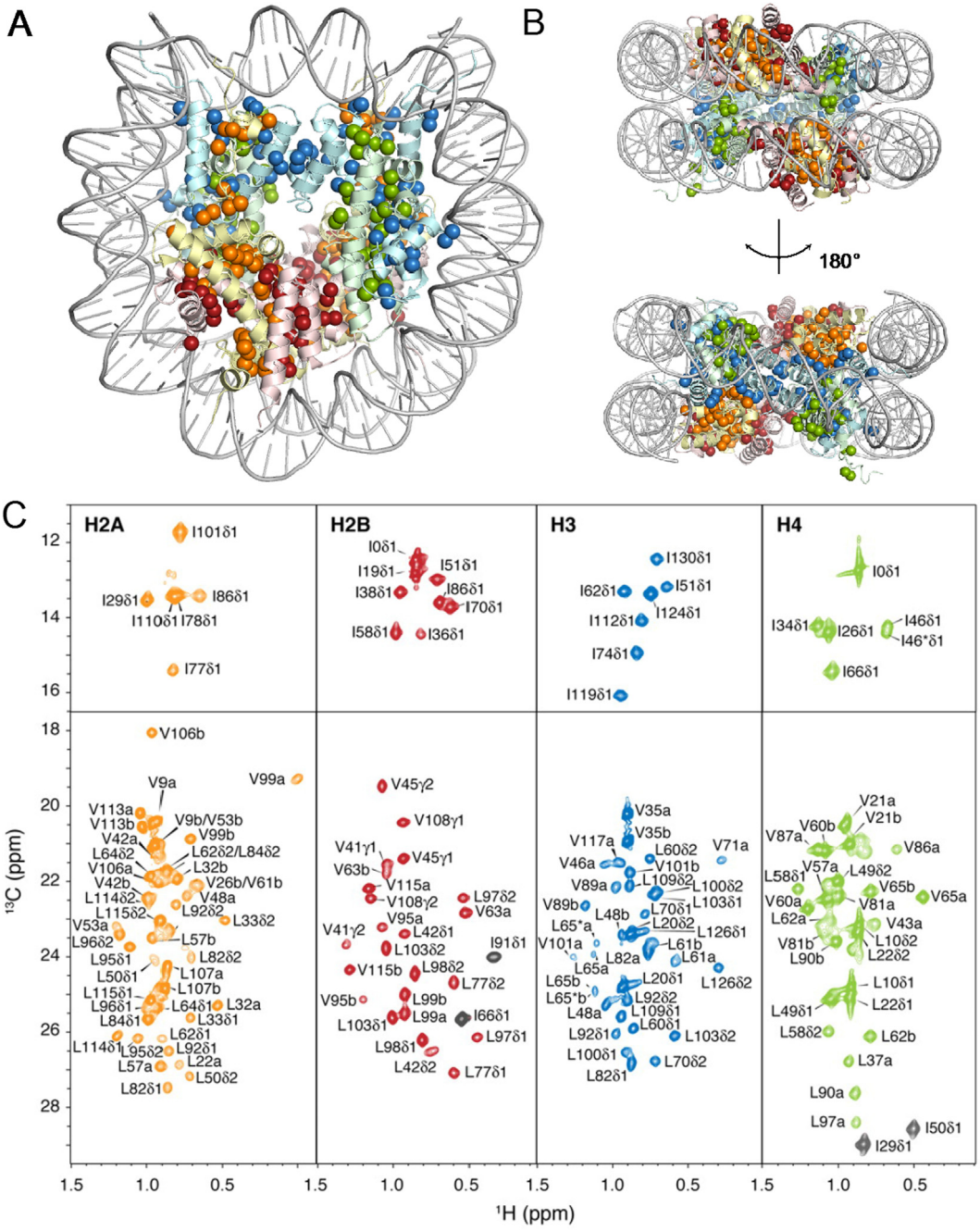

Fig. 4. Histone methyl groups as molecular spies in the MeTROSY approach. Top (A) and side (B) views of the nucleosome (PDB entry 2PYO) showing the position of all ILV-methyl groups (spheres) in the core histones. (C) MeTROSY spectra of ILV-methyl labeled histones H2A, H2B, H3 and H4 in the nucleosome. Stereospecific assignments for leucine and valine are indicated by δ1/δ2 and γ1/γ2 where available, otherwise the two methyl groups are arbitrarily assigned as 'a' or 'b'. The resonances of L65 in histone H3 are significantly broadened due to their close proximity to the DNA, and occur in two sets (labeled L65 and L65*) because the local DNA sequence differs. Reproduced with permission from [97].

technical advantages. One is the ability to observe all ^{15}N and ^{13}C heteronuclei in the histone cores without being restricted to methyl-group containing residue types as in the MeTROSY approach. The second advantage is that the reduced labeling requirements also mean reduced costs for sample preparation. A final advantage is that by using either cross-polarization or scalar coupling-based polarization transfer, spectra can be edited to be sensitive to either the rigid core or mobile tails of the nucleosome. On the other hand, the sensitivity and resolution of ssNMR spectra are generally lower than for MeTROSY solution NMR and titration experiments are not feasible, limiting interaction studies to comparisons of free and bound spectra. Regions with intermediate dynamics appear neither in INEPT or CP-based spectra, and are thus not observable in ssNMR, akin to exchange-broadened residues in solution NMR.

To obtain samples suitable for ssNMR, nucleosomes need to be in a highly dense phase (~0.3–0.5 g/L), not unlike the nuclear nucleosome concentration. The extreme molecular crowding and packing of nucleosomes in these samples prevents overall tumbling, thus allowing cross-polarization methods to work. Two recent studies, one from the Pervushin and Nordenskiöld groups [101] and one from Xiang and Paige et al. from the laboratory of the present authors [102], demonstrate two methods that allow such samples to be obtained. Shi et al. used high concentrations

of Mg^{2+} to precipitate nucleosomes and nucleosome arrays into the NMR rotor. In the Xiang and Paige et al. study the highly dense phase was created by sedimentation of nucleosomes under low Mg^{2+} conditions by overnight ultra-centrifugation [102]. In both cases, highly resolved spectra were obtained, allowing access to structural and dynamical information for nearly all histone core residues.

These two studies also illustrate two different overall approaches. The Pervushin and Nordenskiöld work relied on ^{13}C-detected ssNMR and recorded spectra from 3.2 mm rotors filled with 46 mg of nucleosomes or 1.9 mm rotors containing 15 mg nucleosomal arrays. Recording of 2D ^{13}C-^{13}C correlation maps typically takes overnight under these conditions. The other work relied on ^{1}H-detection, which necessitates fractional deuteration of the isotope labeled histone and the use of a high-speed magic angle spinning (MAS) set-up. Rotors with a 1.3 mm diameter that can be spun at 50–60 kHz MAS were filled through sedimentation using a custom-made filling device with ~2 mg of nucleosomes. Measurement of a 2D ^{1}H-detected NH spectrum typically then requires overnight acquisition, illustrating the sensitivity gain over ^{13}C-detection.

In both strategies, assignment of histone resonances follows well-established ssNMR procedures. For the ^{13}C-detected approach this is based on 2D and 3D NCA, NCO, NCACX, NCOCX and CANCO experiments. For the ^{1}H-detected strategy, triple-resonance 3D CANH, CONH, CA(CO)NH and CO(CA)NH experiments are used. While the Nordenskiöld and Pervushin groups focused their work on histone H4, Xiang and Paige et al. focused on histone H2A. In both cases, the histone of interest was $^{13}C/^{15}N$-labeled, with all other histones and DNA left unlabeled. Histone H2A was also fractionally deuterated to remove ^{1}H-^{1}H dipolar couplings, in particular between the H_N and Hα, to enable ^{1}H detection. In both cases near-complete backbone assignment of the histone core was obtained, and the native histone fold could be confirmed using the assigned chemical shifts.

Internal dynamics of the histones were probed in two different approaches. For H2A, a mostly qualitative assessment of backbone mobility was carried out, simply using the assigned chemical shifts and editing the spectra using either INEPT to observe the histone tails or CP-based transfer to probe the histone core. In a rigorous approach, Shi et al. used 3D DIPSHIFT experiments [147] to derive the order-parameter S^2 for both the Cα-Hα and N-H_N bond vectors for each residue of H4 experimentally.

4.3. Integrative modeling

Structural data obtained by NMR on large macromolecular assemblies such as the nucleosome typically represent either an ambiguous list of residues likely to form an intermolecular interface or sparse intermolecular distance restraints. To translate such structural information into the 3D structures of nucleosome-protein complexes, computational docking approaches, for instance using HADDOCK [148,149] or IMP [150] are needed that integrate the NMR data with other interaction data such as those obtained from mutagenesis, cross-linking or small-angle X-ray scattering (SAXS). Clearly, these modeling approaches require knowledge of the 3D structures of both interaction partners and perform best when conformational changes upon binding are minimal. For a detailed overview of the strategies used in integrative modeling based on NMR data we refer the reader to specialized reviews [151, 152].

5. Unspinning chromatin in high-resolution

Modern sample preparation and NMR techniques have been applied to answer a wide range of questions on the structural conformation and dynamic properties of nucleosomes. In this section, we will review studies on the conformation of the nucleosomal DNA, chaperone-assisted nucleosome assembly, the linker histone and the histone tails. We will discuss several NMR strategies that have been developed to study the real-time deposition of histone tail modifications and the dynamics of the nucleosome core.

5.1. High-resolution nucleosomal DNA studies

The pioneering NMR studies on nucleosomal DNA described in Section 3 lacked the resolution and sensitivity to give detailed information on its conformation. Recently, Xu et al. applied a divide-and-conquer approach to relate DNA sequence to backbone conformation and nucleosome affinity, by cutting 601-DNA into 12 bp fragments and studying these in 2D $^{1}H^{31}P$ correlation experiments [153]. Using the phosphorus chemical shift as a reporter, they conclude that the sequence-dependent phosphate backbone conformation determines the minor groove width and that the minor groove width profile of 601-DNA fragments is highly similar to the variations in minor groove width observed in the NCP crystal structures. In a follow-up paper they extended this method with internucleotide distances from NOE connectivities and RDCs, which allowed the characterization of additional internucleotide parameters like roll, twist and slide to further determine the intrinsic properties of nucleosomal DNA fragments in solution [154]. Such high-resolution studies of the DNA in the context of nucleosomes is as yet beyond the current state-of-the-art.

5.2. Chaperone-assisted nucleosome assembly

Nucleosomes are assembled by deposition of H3-H4 tetramers and H2A-H2B dimers onto the nucleosomal DNA. *In vivo*, this process is guided by histone chaperones to prevent the formation of non-native complexes [155]. In addition, these chaperones play an important role in the incorporation of histone variants. In order to understand the process of nucleosome assembly, several NMR studies have focused on the structural and dynamic properties of these histone complexes and their interactions with chaperones.

The smallest stable subsystem within the nucleosome is the H2A-H2B dimer (25 kDa). The solution structure of isolated H2A-H2B dimers, obtained from backbone chemical shifts using CS-Rosetta [156], shows that, while the core region is well-folded, several well-defined regions of the dimer in the nucleosome are disordered in the isolated dimer (Fig. 5A) [100]. Presumably, these regions become rigidified and folded upon interaction with H3-H4 or the DNA in the context of the nucleosome. Interestingly, H/D and solvent exchange and $^{1}H^{15}N$-NOE measurements indicate that these regions do retain some residual structure. Importantly, regions of the histone core that are recognized by chromatin factors and histone chaperones are relatively well-defined, including the positively charged DNA binding surface and a hydrophobic pocket in the H2B α1-α2 loop that are recognized by several histone chaperones.

Histone chaperones show a wide range of histone binding modes ranging from interactions between folded protein domains to purely disordered interactions. NMR is suitable for deciphering all these interaction types, and in particular those that involve disorder. For studies on folded chaperones, traditional structure determination and interaction studies are very suitable, as for instance in the study of H3 chaperone Asf1a [157]. A nice illustration of using H/D exchange by NMR to determine the histone binding interface is offered by Su et al. [158] for histone chaperone Vsp75. Here, NMR spectra offer atomic resolution mapping of increased protection from deuterium exchange in the histone-chaperone complex. Another class of chaperones contains intrinsically disordered regions that become folded only upon binding.

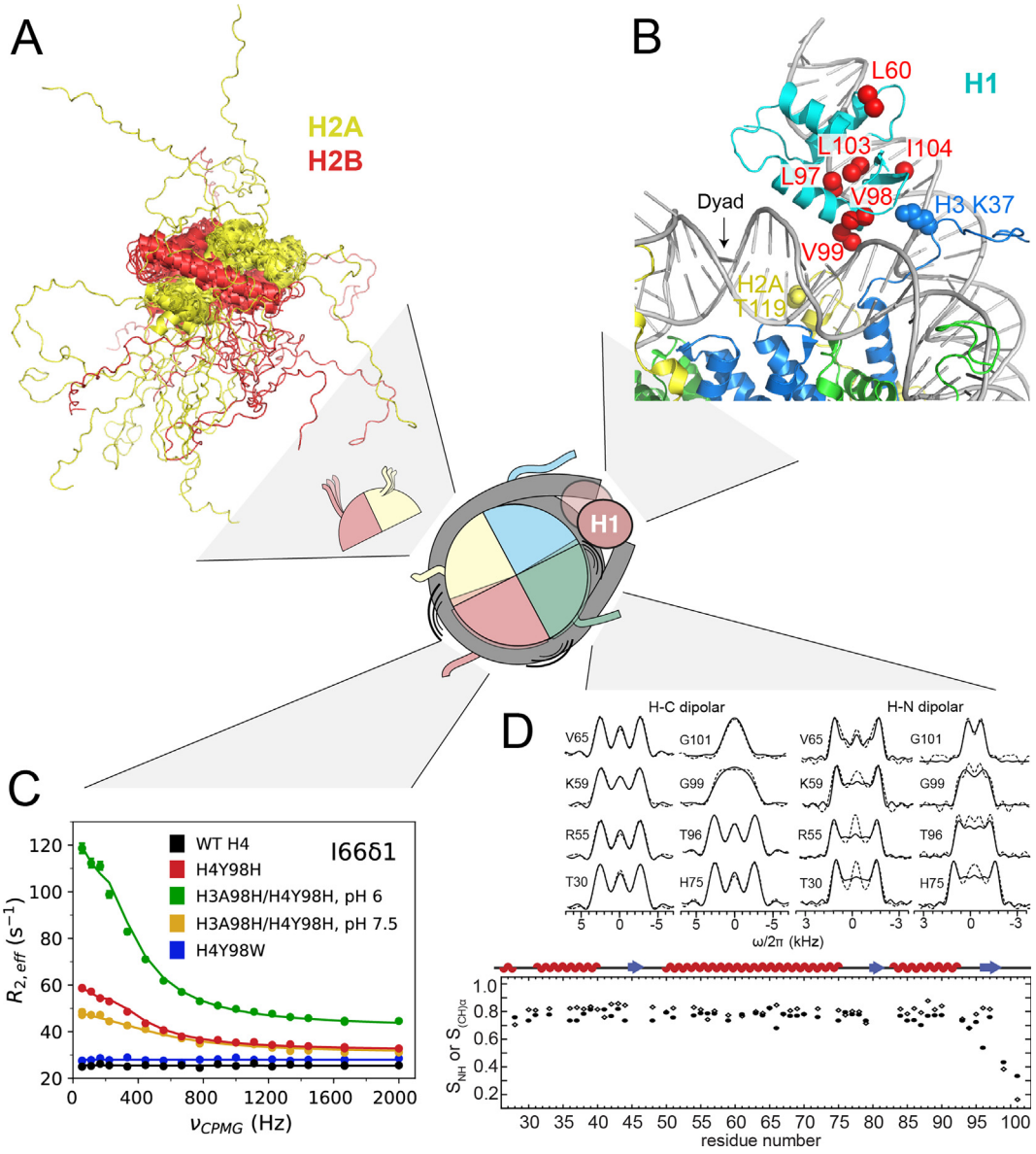

Fig. 5. Schematic overview of NMR studies on histone structure and dynamics in the nucleosome. (A) Solution structure of the H2A-H2B dimer obtained by NMR (ensemble of 10 best scoring solutions based on CS-Rosetta, PDB entry 2RVQ), showing a native core histone fold as well as extensive unstructured tail regions. (B) NMR structure of the chromatosome based on MeTROSY PRE effects from spin-labeled H3K37 (blue spheres) or spin-labeled H2AT119 (yellow spheres) on ILV methyl groups in H1 (red spheres). Figure adapted with permission from [164]. (C) MeTROSY CPMG relaxation dispersion profiles of ILV-labeled histone H2B (recorded at 600 and 800 MHz) show increasing slow timescale dynamics upon destabilizing mutations in H3 and H4. Figure reproduced with permission from [165]. (D) Histone H4 dynamics obtained by fitting of ^{1}H-$^{13}C\alpha$ and ^{1}H-^{15}N dipolar line shapes from 3D DIPSHIFT ssNMR experiments at 800 MHz (top) to derive $S_{(CH)\alpha}$ and S_{NH} order parameters (bottom). Figure reproduced with permission from [101]. Color coding of histones and DNA as in Fig. 1.

Here, NMR has been used for the structure determination of several chaperone-histone variant complexes, using a chaperone-variant chimera in order to obtain a stable complex and allow a traditional NOE-based structure calculation [126,159,160]. Many chaperones contain stretches of acidic residues. The importance of such regions for the recruitment and binding of histones was recently demonstrated by NMR for nucleoplasmin [161]. Corbeski et al. showed from a series of NMR interaction studies that sparse aromatic residues in such acidic domains can be critical to obtain the functional, specific binding mode [162]. At the other extreme is the spectacular case of histone H1 chaperone ProTα [163]. This intrinsically disordered chaperone interacts with the disordered tail of H1 with picomolar affinity. Remarkably, NMR studies revealed that both proteins fully maintain their flexibility and disorder in the complex. The large opposite net charge and extended interaction surfaces of the two proteins allow an extremely tight interaction without requiring a defined, structured binding mode.

While most studies have focussed on structures of chaperone-histone complexes, NMR can also be used to determine the interaction dynamics quantitatively. This was nicely illustrated for the Chz1 chaperone using CPMG relaxation-dispersion experiments to determine the dissociation rate of the complex [159]. Similar studies on chaperone-histone complexes in the presence of DNA or histone-DNA complexes may offer detailed insights into the nucleosome assembly process.

5.3. The chromatosome: orientation of histone H1

Recent studies on the structure of the chromatosome revealed the orientation and binding mode of histone H1 on the nucleoso-

mal DNA. In 2013, the Bai lab reported an NMR-based structural model of the complex in which the globular domain of *Drosophila* H1 bridges the nucleosome core and the linker DNA by binding close to, but just off-center from, the central base pair at the dyad [164]. Using the MeTROSY approach, they performed paramagnetic relaxation enhancement (PRE) experiments to obtain long-range distance information on the location and orientation of H1 and model its position on the nucleosome using computational docking (Fig. 5B). Such an off-dyad binding mode of the human linker histone is also reported by Song et al. [99], who solved the 11 Å structure of a 30 nm chromatin fiber by cryo-electron microscopy (cryo-EM). Interestingly, the first near-atomic resolution crystal structure of the chromatosome as well as a recent cryo-EM study showed on-dyad binding of chicken histone H5 and Xenopus histone H1, respectively [166,167]. NMR and spin-labeling experiments revealed that five mutations in the globular domain of H5 into the corresponding residues in H1 can change the binding mode from on-dyad to off-dyad [168]. The linker histone binding mode may be crucial in determining the conformation of higher order chromatin structure, which is emphasized by a recent study showing that the disordered H1 C-terminal tail promotes DNA compaction depending on its phosphorylation state [169].

5.4. Mobility of histone tails in a chromatin context

In the past ten years, several sophisticated NMR studies were published reporting on the structure of the histone tails in the context of the full nucleosome and nucleosome arrays. Using MeTROSY and PRE experiments, the Bai lab showed that the basic patch in the tail of histone H4 folds onto the nucleosome core and that this interaction can be disrupted by acetylation of lysine 16 [170]. The structure of the H3 tail in condensed nucleosomal arrays was addressed by the Bai lab using an H/D exchange experiment. After subjecting the arrays to H/D exchange, the histones are extracted in DMSO to quench the reaction and dissolve the aggregates, before $^{1}H^{15}N$ HSQC detection of the backbone amide signals of H3 [171]. Several tail residues of H3 showed slower exchange than expected for a purely unfolded state, suggesting that the H3 tail forms stable structures in condensed chromatin. Later, the Jaroniec lab used ssNMR to probe the structural and dynamic properties of the H3 and H4 histone tails in nucleosome arrays at varying degrees of Mg^{2+}-induced compaction [98]. In contrast to the earlier report, they find that both the H3 and the H4 tail are highly dynamic in nucleosome arrays as they are observed in INEPT-based ^{13}C-detected ^{1}H-^{13}C correlation spectra. The apparent difference from the Bai study may in part be due to the different readout chosen. In addition, the authors suggest that the histone tails could remain partly protected from solvent exchange while highly flexible. An insightful third study from the Selenko and Fischle labs added another dimension to the question of histone tail conformation. A series of solution NMR experiments, including ^{15}N-based relaxation measurements, showed that H3 tail flexibility in nucleosomes decreases upon inclusion of linker DNA and linker histone, which can be counteracted by introducing charge-modulating PTMs [172]. It was also shown that the H3 tail has intrinsic DNA binding affinity, indicating that the H3 tail transiently interacts with nucleosomal (linker) DNA. These transient, PTM-dependent interactions may not only stabilize higher order chromatin, but also regulate accessibility to protein binding (see below). Recent ssNMR work showed that the H2A N-terminal tail has different chemical shifts in the nucleosomal context compared to the dimer, indicating it may be similarly bound to the DNA [102]. Furthermore, peak splitting for several resonances matched the asymmetric DNA sequence close to the H2A tail location. The importance of PTMs for chromatin structure is further illustrated by a recent study on the effect of histone ubiquitination. This study employed H/D exchange in combination with NMR to map the interaction surface of H2BK120-conjugated ubiquitin in the context of 12-mer nucleosome arrays [173]. A small acidic patch on ubiquitin was found to be involved in chromatin decompaction by acting as a dynamic wedge between nucleosomes in the array.

5.5. Following nucleosome modification in real-time

Apart from structural and dynamics studies, NMR can also be used to study the modification of histones in real time. For example, binding affinities and conversion rates for proline isomerization by the PPIase domain of Frp4 were determined by NMR for several H3 peptides by measuring the intensities of the exchange cross-peaks in $^{1}H^{1}H$ NOESY experiments [174]. In an interesting approach, the Selenko and Schwarzer labs determined the activity of deacetylases and acetyl transferases on H4 tail peptides in HeLa nuclear extracts [175]. Incorporation of NMR-active isotopes at selected lysine backbone and sidechain positions permitted the *in situ* observation of multiple acetylation events in parallel, in a time-resolved and quantitative manner. A subsequent study by the same lab used an elegant isotope labeling and histone purification scheme to study asymmetric modification of histones in the context of the nucleosome [176]. Exploiting orthogonal, cleavable affinity tags, asymmetric H3-H4 tetramers were obtained in which one H3 chain is ^{15}N-labeled and the other ^{13}C-labeled. With this approach, PTM crosstalk in *cis* (within one H3 tail) and in *trans* (between two H3 tails in the same nucleosome) was analyzed by monitoring either a ^{13}C- or a ^{15}N-edited spectrum during enzymatic deposition of a second PTM.

5.6. Nucleosome dynamics

The nucleosome is subject to several dynamic processes, including assembly/disassembly, incorporation of histone variants and nucleosome remodeling, that are likely to require a certain amount of nucleosome deformation and plasticity. While less apparent than the flexibility of the histone tails, the dynamics of the nucleosome core is of particular interest to understand these processes. Two recent solution NMR studies show that the nucleosome core is indeed inherently dynamic and that this property is essential for successful remodeling. The Narlikar lab used MeTROSY NMR using nucleosomes containing ILV-labeled H4 to probe deformation of the histone core upon remodeling by SNF2h [177]. They found that binding of SNF2h induces line broadening for several buried methyl groups, implying a conformational change of the histone core. This change could be mapped to ILV residues in the H3-H4 interface. Constraining this interface by site-specific cross-linking strongly inhibited nucleosome remodeling by SNF2h, suggesting that plasticity of the H3-H4 dimer interface is required for function. Similarly, a study from the Luger and Kay labs showed by MeTROSY CPMG relaxation dispersion measurements that destabilizing mutations in the dimer-tetramer interface between H2A-H2B and H3-H4 lead to significant (~8%) populations of 'excited' conformational states (Fig. 5C) [165]. While the ground state structure is unaffected by the mutation, the presence of these higher-energy conformations indicates that the H2A-H2B dimer can sample a more loosely associated state on a millisecond timescale. Such increased plasticity of the nucleosome could also be exploited by histone variants or PTMs to modify nucleosome function.

These studies are complemented by two recent ssNMR studies reporting on histone dynamics in the nucleosome core. The first study, by Xiang and Paige et al. from the present authors' laboratory, used ^{1}H-detected ssNMR to probe the structure and dynamics of H2A in sedimented nucleosomes [102]. Based on observation of resonances in either INEPT or CP-based spectra together with backbone chemical shifts values, a clear trend in dynamics was

observed: from highly flexible N- and C-terminal tails observable in the scalar-based spectra, to a rigid histone fold core. Notably, part of the αN helix and the C-terminal docking domain showed increased flexibility. In an elegant and thorough study, the Pervushin and Nordenskiöld labs determined the dynamics of histone H4 in the nucleosome to that in a 12-mer nucleosome array [101]. By careful measurement and fitting of the $^{1}H_{N}$-^{15}N and $^{1}H\alpha$-$^{13}C\alpha$ dipolar line shapes in N-CA correlation maps, order parameters and thus the extent of μs-ms backbone motions of H4 could be determined (Fig. 5D). The most dynamic regions in nucleosome and nucleosome arrays include residues involved in histone-DNA contacts, which could mean that these motions are important for DNA accessibility and nucleosome mobility.

6. Nucleosome-protein interactions

One of the defining features of the nucleosome is its function as a landing and binding platform for a wide array of proteins that control chromatin biology (see Section 2). Structurally these interactions can be classified according to their interaction site on the nucleosome: the nucleosomal DNA, the histone tail or the nucleosome core surface [178]. Nucleosome-binding proteins show a large variety of binding sites and modes, including low affinity or multivalent binding, and can be mediated through unstructured regions, relying predominantly on electrostatic interactions. Each of these interaction types poses their own requirements and challenges when addressing them by NMR, and these are reviewed in this section.

6.1. Structural basis of the histone code

Since histone tails are generally considered to behave as independent units due to their flexibility, protein interactions with the tails have primarily been studied in a divide-and-conquer approach using histone peptides. Importantly, this allowed for easy implementation of PTMs such as lysine acetylation or methylation at defined positions through the use of solid phase peptide synthesis, as well as the use of relatively straightforward NMR methods to study the interactions of these peptides with protein domains.

In the earliest demonstration of specific readout of a histone modification, solution NMR was used to determine the first structure of a bromodomain. Titration experiments showed that this domain binds specifically to histone H4 tails carrying an acetylated lysine, and allowed mapping the location of the binding cleft onto the protein surface [90]. Similarly, the specific readout of trimethylated lysine was first demonstrated using NMR titration experiments [179]. The structure of the reader domain bound to a methyllysine histone peptide, determined both by NMR [92] and X-ray crystallography [91], showed that the recognition relies on the interaction of the trimethyl group with a cavity containing aromatic residues, coined the aromatic cage.

These NMR-based interaction studies provided crucial evidence and support for the so-called histone code hypothesis, as they revealed the structural basis and mechanism for its read-out [180–182]. Such studies have also been instrumental in establishing the structural basis for the combinatorial readout of histone modifications, first demonstrated in the Allis lab by identifying a K9 methylation/S10 phosphorylation switch for the binding of heterochromatin protein HP1 to the H3 tail [183]. This multivalent recognition of histone modifications can also be mediated by tandem reader domains, in which both domains each bind to a specific histone modification [184,185].

A similar set-up of NMR titrations of an isotope-labeled reader domain with modified histone peptides, often together with NMR solution structures of free and complexed proteins, has been used extensively since. Two recent studies have extended this by using a spin-labeled or ^{19}F-labeled version of the reader domain to characterize the histone interaction [186,187].

An as yet scantly covered area is the protein interactome of linker histone H1. The Thomas lab showed by NMR that the acidic tail of HMGB1, a member of the class B high mobility group proteins, interacts with the basic tail of H1, which may thereby promote the replacement of H1 with HMGB1 at distinct nucleosomal sites [188].

6.2. Histone tail interactions in the nucleosomal context

The assumption of histone tails as completely independent units ignores the nucleosomal context in which they occur. Thus, the peptide-based studies described above potentially miss out on additional contributions of nucleosomal DNA or histone cores to the interaction, or the competing effect of the histone tail–DNA interaction that may reduce the availability of binding competent states (see Section 5.4).

A study from the Zweckstetter lab was the first to address this issue focusing on the interaction of heterochromatin protein HP1 with nucleosomes carrying the H3K9me3 modification [132]. Comparing the interaction with modified nucleosomes and modified peptides, they found that the CSPs in HP1 were smaller in the nucleosome case, indicating that the nucleosomal context reduces the binding affinity two-fold. Importantly, the same set of HP1 residues showed CSPs and the perturbations were in the same spectral direction, evidencing that the binding mode is maintained between peptide and nucleosome. Notably, being bound to the histone tail, HP1 remains rather mobile in the complex, which is clear from the successful observation of the protein by amide backbone TROSY and further proven by relaxation experiments. Interestingly, a second ‘explorative’ binding mode was uncovered using unmodified nucleosomes in which HP1 weakly interacts with DNA.

Two recent studies shed more light on the origin of the inhibitory effect of the nucleosomal context. Gatchalian et al. investigated the interaction of nucleosomes with a paired reader domain, a construct containing two PHD domains that can each bind the H3 histone tail [189]. Affinity and NMR measurements showed that nucleosome binding is impaired approximately six-fold compared to H3 peptide binding, while the binding mode and the relative binding order of the PHD domains is maintained. Additional NMR experiments showed that one of the PHD domains in particular is repelled by the nucleosomal DNA. Combined with partial occlusion of the H3 tail due to DNA interaction, this likely accounts for the reduced affinity. Recently, Morrison et al. found a very pronounced inhibition of the interaction between the BPTF PHD finger reader domain and the H3K4me3 mark in the nucleosomal context [190]. From a series of NMR titration experiments it was shown that the H3 tail transiently and dynamically interacts with nucleosomal DNA also in the nucleosome core particle, in the absence of any linker DNA. They further showed that the inhibition of tail-reader binding can be released by H3 modifications that weaken its interaction with the DNA (Fig. 6A).

The nucleosomal context can also promote histone tail interactions, as was shown for the recognition of the H3K36me mark. This modification site, K36, is close to the point where the H3 tail exits the core particle and thus any reader domain of H3K36me will be close to the nucleosomal DNA. This was first shown for the PSIP1 PWWP reader domain by Van Nuland et al. [130]. NMR titration experiments showed that this reader domain binds with high millimolar affinity to a modified peptide and that this very weak interaction is completely dependent on the presence of the modification. In the context of modified nucleosomes however, a low micromolar affinity was found, corresponding to a dramatic increase in affinity of four orders-of-magnitude. Here, MeTROSY titration experiments were used to determine the microscopic affin-

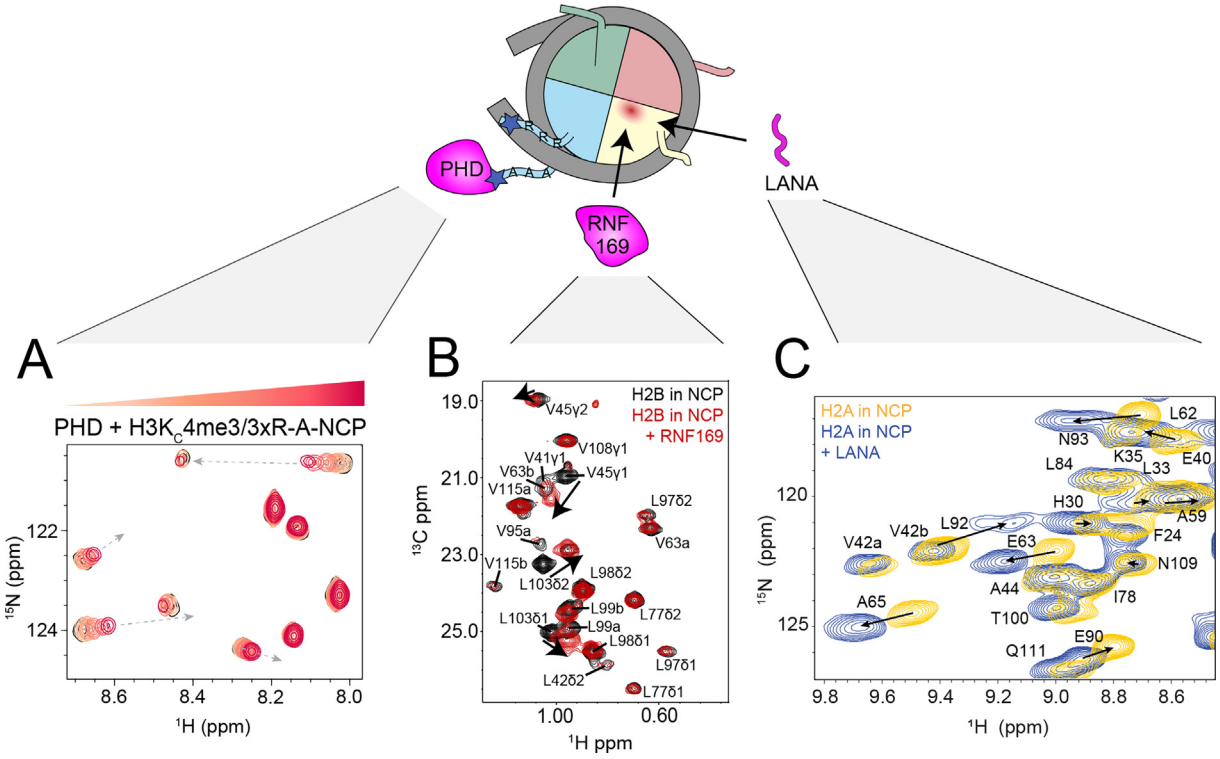

Fig. 6. Schematic overview of nucleosome-protein interaction studies by NMR. (A) Recognition of the trimethylated K4 residue in the histone H3 tail (H3K4me3) by the PHD reader domain as probed by NMR titration experiments. Addition of nucleosomes containing three arginine to alanine mutations in the H3 tail and a cysteine-based mimic for H3K3me3 (H3K$_C$4me3) result in clear and specific chemical shift changes for the reader domain. These mutations were necessary to weaken the tail-DNA interactions and increase the affinity of the nucleosome for the PHD reader domain. Figure reproduced with permission from [190]. (B) Recruitment of RNF169 to nucleosomes ubiquitinated at H2A K13 or K15 depends on specific interaction of its UDM2 domain with the H2A-H2B acidic patch. Addition of this domain to ILV-methyl labeled H2B in H2AK13$_C$Ub-NCPs resulted in several significant CSPs of acidic patch residues (indicated by arrows in the spectrum). Figure reproduced with permission from [133]. (C) Mapping of the LANA-peptide binding interface on the nucleosome surface by ssNMR 2D NH spectra (800 MHz) on sedimented, free (yellow) and LANA-bound nucleosomes (blue) containing labeled H2A. Figure reproduced with permission from [102]. Color-coding and symbols of the cartoon are as in Fig. 1, acidic patch is indicated in red.

ity at the H3 tail site by fitting of the line shapes of perturbed H3 ILV residues. The structural model based on NMR and mutation data reveals how the PWWP reader domain is optimized to recognize the nucleosomal context of this modification by engaging simultaneously to the methylated histone tail and the DNA backbone.

In a study of Musselman et al. similar enhancement of affinity in the nucleosomal context was observed for the recognition of H3K36me by PHF1 Tudor domain [191]. Interestingly, a high flexibility of a reader-nucleosome complex was observed, permitting the use of amide-observed TROSY NMR to map the binding surface on the reader domain. The structural model again showed that the reader domain can interact with histone tail and DNA simultaneously, rationalizing the enhanced affinity. This study also points out the potential impact of protein interactions on nucleosome organization or stability, since both NMR and FRET data revealed a highly dynamic complex and partial DNA unwrapping. Protein binding may cause increased nucleosome opening and DNA breathing, opening up otherwise occluded binding interfaces on the DNA or histone octamer. This was also hypothesized by Richart et al., who show that the chromoshadow domain (CSD) of HP1α binds to histone H3 on a site that is located just within the nucleosome core [192].

6.3. Docking onto the nucleosome core

In addition to histone tails, the histone core also features several sites for post-translational modifications that could act as a binding site for reader proteins [193–195]. Many proteins have been identified over the last decade that bind to the nucleosome core. Apart from PTMs in the core, a negatively charged patch on the surface of the H2A-H2B dimer, often referred to simply as the acidic patch, has emerged as a prominent docking platform for many proteins that regulate chromatin function [24]. Invariably, these proteins use an arginine residue to anchor to the acidic patch, without sequence motif or structural motif apart from hydrogen bonding. In favorable cases these interactions can be studied in a histone dimer context, but in general it requires the whole nucleosome to prevent missing out on detection of synergistic interactions with other histones or DNA. In addition, the opposite charge and different salt stability of the nucleosome and dimer may affect complex stability to a great extent.

The first study to show that NMR can resolve the structural basis of nucleosome-protein complexes was a collaboration between the Kay and Bai labs [97]. This study pioneered the use of MeTROSY for nucleosomes and reported the assignments of the ILV methyl groups in all four histones (see Section 4.2). Using these signals as probes, the interaction with the architectural chromatin factor HMGN2 was studied. This intrinsically disordered protein was known to anchor to nucleosomes but the exact binding mode and interaction surface on the nucleosome were unclear (see also Section 3.4). On the basis of observed CSPs and saturation transfer experiments, the binding site of HMGN2 could be mapped to the acidic patch on the nucleosome surface. Additional mutational analysis revealed the crucial contribution of two conserved arginine residues to mediate the interaction. A series of PRE measurements using spin-labeled versions of HMGN2 revealed that its lysine-rich region is anchored to the nucleosomal DNA around the H3 tail exit site, highlighting the importance of studying such interactions in the nucleosomal context. The NMR and mutation data-driven structural model shows how this chromatin factor

'staples' nucleosomes through the two binding sites and thus orients its regulatory C-terminal domain to antagonize with linker histone binding, potentially destabilizing higher order chromatin.

Using the same MeTROSY approach, the Bai lab determined how centromeric protein CENP-C recognizes nucleosomes containing histone H3 variant CENP-A to initiate assembly of the kinetochore during cell division [196]. NMR experiments showed that a disordered segment of CENP-C is responsible for nucleosome binding. Through a combination of PRE data from spin-labeled versions of CENP-C and titration experiments, both short- and long-range contacts between CENP-C and histones in the nucleosome could be mapped. This pointed to a simultaneous engagement of CENP-C to a hydrophobic region in the CENP-A tail and the acidic patch of H2A and H2B, which was confirmed in a crystal structure [196].

A similar dual recognition mode was found for the recruitment of E3-ligase protein RNF169 binding to ubiquitylated nucleosomes in a study from the Kay lab [133]. Here, the interaction depends on the ubiquitin-dependent recruitment module (UDM2) of RNF169, again containing a disordered region responsible for nucleosome binding. Ubiquitinated nucleosomes were prepared by converting G76 of ubiquitin and K13 of H2A to cysteine residues, conjugating them through sidechain-sidechain disulfide linkage before reconstitution. Nucleosomes contained either ILV-methyl labeled H2A, H2B or ubiquitin. MeTROSY-based relaxation measurements showed that the ubiquitin moiety is flexibly attached to the nucleosome. A series of titration experiments demonstrated that UDM2 binds to a hydrophobic patch on ubiquitin, and to the acidic patch of the nucleosome using two basic regions with arginine residues (Fig. 6B). Notably, a series of nucleosome and RNF169 mutants was screened by NMR to show not only the impact on binding affinity but also on binding mode, which proved crucial to dissect the different interactions to ubiquitin and the nucleosome core. The Mer lab studied the same interaction in a divide-and-conquer approach. First, the structure of a chimeric H2AK15Ub-H2B fusion protein in complex with UDM2 was determined based on intermolecular NOE and PRE restraints, together with backbone dihedral angle restraints [125]. Together with additional SAXS data on the nucleosomal complex, the structure of nucleosome-bound RNF169 was modeled. Together, the studies described here illustrate that synergistic interactions are pervasively present in nucleosome-protein interactions.

The present authors' lab recently demonstrated the potential of ^{1}H-detected ssNMR in studying nucleosome-protein interactions [102]. A key advantage here is the ability to observe all backbone NH correlation maps as in standard solution NMR, thus allowing the use of all non-proline amides as reporters on the interaction. ^{1}H detection is required to exploit the sensitivity of the ^{1}H spin to changes in the chemical environment upon binding. Using the LANA-nucleosome interaction as a proof of principle, this study showed that it is possible to prepare a ssNMR sample of a complex through co-sedimentation of nucleosomes with the binding protein, in this case the LANA-peptide. Since titrations are not feasible in this set-up, CSPs have to be determined from a comparison of apo and bound state NH spectra. For unambiguous assignment of bound state resonances, a 3D HNCA experiment was recorded on the bound state. The changes in HNCA chemical shifts thus obtained accurately mapped the LANA binding site (Fig. 6C) and further confirmed that binding does not induce changes in H2A conformation from the lack of Cα change, in agreement with the previously published crystal structure [95].

7. Conclusions and outlook

Here, we have reviewed the contribution of NMR to nucleosome research, from the early work in the late 1960s pioneering structural investigations of histones and the conformation of nucleosomal DNA to the characterization of numerous reader-histone tail interactions and the most recent state-of-the-art NMR studies elucidating high-resolution structures of nucleosomes and their complexes with interacting proteins. Advances in both sample preparation and NMR methodology have gone hand-in-hand to deliver increasingly high-resolution information on nucleosome structure, dynamics and interactions. The development of MeTROSY-based solution NMR and advances in biomolecular ssNMR have been instrumental in pushing these studies to the level of the whole nucleosome.

A major open challenge for NMR is the assessment of DNA conformation and dynamics in the nucleosome. The molecular size and limited chemical shift dispersion of nucleosomal DNA essentially render the DNA invisible to either solution or solid-state NMR. Future experiments employing segmental or site-specific labeling [197] might overcome some of these challenges and allow direct and high-resolution detection of nucleosomal DNA. Alternatively, site-specific incorporation of paramagnetic tags into DNA [198] could enable indirect monitoring of dynamic changes in histone-DNA interactions. While it will require a formidable effort, such methods may open the door to future studies on the contribution of DNA in nucleosome-protein interactions, in particular in remodeling, the conformation and flexibility of non-positioning, genomic DNA sequences, or the impact of DNA modifications.

Focusing on histones and nucleosome-binding proteins, we expect that on-going developments in NMR methodology will enhance the study of nucleosome-protein interactions in particular. Application of intermolecular PCS holds great promise to reveal quantitative long-range interaction data, complementing CSP-based identification of binding surface and sparse short-range NOE data. In addition, the sedimentation approach will be useful to characterize the structures of nucleosome-bound proteins and thus reveal conformational changes upon binding by ssNMR.

An intriguing question in the chromatin field concerns the impact of DNA sequence on nucleosome stability and dynamics. NMR studies on nucleosomes containing genomic DNA sequences will be highly instructive to expose the extent and timescales of histone dynamics in such nucleosomes, at atomic resolution. Moreover, the use of genomic DNA sequences also enables the incorporation of native binding sites for (pioneer) transcription factors to study their binding to the nucleosome [199].

It will be exciting to see how the study of these native-state interactions can be extended to higher order chromatin systems. The wide applicability of NMR to different sample phases, from dilute solution to condensed phases and cellular systems, allows investigation of biomolecular systems at different levels of complexity. We envisage that the combined use of solution, solid-state and in-cell NMR methods has the potential to enable multi-scale studies of chromatin systems and nucleosome-protein interactions, ranging from mononucleosomes to nucleosomal arrays to *in vivo* chromatin. By building on the intrinsic atomic resolution of the NMR signal and its sensitivity to molecular motions, NMR offers a unique perspective on the dynamic landscape of nucleosomes and their interacting proteins, which is crucial for our understanding of chromatin function.

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Glossary of Abbreviations

ATP: adenosine triphosphate
bp: base pair
CEST: chemical exchange saturation transfer
CP: cross-polarization
CPMG: Carr-Purcell-Meiboom-Gill sequence
Cryo-EM: cryo-electron microscopy
CSP: chemical shift perturbation
DNA: deoxyribonucleic acid
EM: electron microscopy
HMG: high mobility group proteins
HMQC: heteronuclear multiple quantum correlation
HSQC: heteronuclear single quantum correlation
INEPT: insensitive nuclei enhanced by polarization transfer
MAS: magic angle spinning
MeTROSY: methyl transverse relaxation optimized spectroscopy
MNase: micrococcal nuclease
NCP: nucleosome core particle
NMR: nuclear magnetic resonance
NOESY: nuclear Overhauser effect spectroscopy
PAGE: polyacrylamide gel electrophoresis
PCR: polymerase chain reaction
PCS: pseudo-contact shift
PDB: Protein Data Bank
PRE: paramagnetic relaxation enhancement
PTM: post-translational modification
RDC: residual dipolar coupling
RNA: ribonucleic acid
SAXS: small angle X-ray scattering
ssNMR: solid-state nuclear magnetic resonance
TOCSY: total correlation spectroscopy