Studies on the mechanism of nucleosome remodeling by RSC and SWI/SNF

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THÈSE

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Manu Shubhdarshan Shukla
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Etudes sur le mécanisme de remodelage des nucléosomes par RSC et SWI/SNF

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Thèse préparée au sein du laboratoire
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To,

My Grand Pa

Late Shri Uday Narain Shukla

Acknowledgements

I have always believed that any personal achievement has many contributions from different quarters. As I reflect back, I see that without these it would not have been possible to realize the goal of completing my thesis. I wish to take this opportunity to thank those who have been instrumental in making this possible.

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Table of Contents

CHAPTER II: *Manuscript communicated-* **A Two-Step Mechanism for Nucleosome Remodeling by RSC: Initial Formation of a Remosome Containing ~180-190 bp DNA Followed by Sliding**

Index of Figures and Tables

13

Thesis Details

A.   Title

a.   Titre en français

Etudes sur le mécanisme de remodelage des nucléosomes par RSC et SWI/SNF

b.   Title in english

Studies on the mechanism of nucleosome remodeling by RSC and SWI/SNF

B.   Abstract

a.   Résumé en français

Dans les cellules eucaryotes l'ADN nucléaire est organisé sous la forme de chromatine, dont l'unité de répétition est le nucleosome. En règle générale, la chromatine est considérée comme répressive pour les processus nécessitant un accès à l'ADN tels que la transcription, la réplication ou la réparation. Le nucléosome représente une forte barrière pour des protéines nécessitant l'accès à l'ADN. Pour surmonter cette barrière, la cellule a développé des méthodes variées, dont la plus importante semble être le remodelage des nucléosomes dépendant de l'ATP. Une propriété commune à tous ces facteurs de remodelage est leur capacité de repositionner les nucléosomes le long de l'ADN.

Dans ce travail, nous avons étudié le mécanisme de déplacement des nucléosomes par RSC et SWI/SNF, deux facteurs de remodelage de levure bien caractérisés. Nous avons combiné des approches basées sur la visualisation à haute résolution, notamment la microscopie à force atomique (AFM) et la cryo-microscopie électronique, avec des approches nouvelles à pointe de la biochimie et de la biologie moléculaire.

Nous avons montré que la mobilisation des nucléosomes par RSC ou SWI/SNF implique des espèces réactionnelles intermédiaires métastables dont l'existence et la structure étaient jusqu'alors inconnues. Ces particules nucléosomales, que nous avons nommé 'remosomes', possèdent certaines propriétés structurales distinctes des nucléosomes canoniques. En particulier, les 'remosomes' contiennent ~180 pb d'ADN associées à l'octamère d'histones au

lieu de 147 pb pour les nucléosomes canoniques. En utilisant, l'empreinte à la DNase I nous avons montré que le 'remosome' représente un ensemble de structures multiples caractérisées par un enroulement fortement perturbé de l'ADN sur l'octamère d'histones. Pour caractériser ces 'remosomes' avec une grande précision, nous avons mis au point une nouvelle technique « *one pot in gel assay* » qui consiste à cartographier toutes les 10 pb l'accessibilité d'une enzyme de restriction au 'remosome' fractionné. L'application de cette technique a révélé que le profil de l'accessibilité du 'remosome' est très différent de celui du nucléosome. Alors que celui du nucléosome peut être extrapolé par une fonction de type hyperbolique, le profil du 'remosome' est ajusté par une fonction parabolique.

Nous avons voulu répondre à la question du mécanisme de l'inhibition de la mobilisation du nucléosome variant H2A.Bbd par SWI/SNF. En utilisant les techniques décrites plus haut sur des nucléosomes variants ou chimériques (contenant des délétions ou translocations de domaines d'histones) nous avons montré que le domaine d'accrochage ('*docking domain*') de l'histone H2A est essentiel pour la mobilisation des nucléosomes. Nous avons aussi montré que l'incapacité du nucléosome à glisser est due à la génération d'états intermédiaires 'remosomes erronés', distincts de ceux apparaissant dans le cas du nucléosome conventionnel.

b. Abstract in English

In eukaryotic cell the DNA is organized in the nucleus in the form of chromatin, the fundamental unit of which is called as the nucleosome. Organization of DNA into the nucleosomes presents a strong barrier for various processes which require access to the DNA like transcription, replication and repair. To overcome this problem cells utilize a variety of methods, ATP dependent chromatin remodeling being one of the most important of them. A common feature of all the remodelers is that they are able to reposition the nucleosomes along the DNA at the expense of ATP.

In the present work, we have studied the mechanism of nucleosome mobilization by RSC and SWI/SNF, two well characterized remodelers from yeast. A combinatorial approach was employed using high resolution microscopy namely Electron cryo-Microscopy (EC-M) and Atomic Force Microscopy (AFM) together with novel biochemical approaches. We have shown that the nucleosome mobilization by RSC and SWI/SNF involves hitherto unknown

intermediate structures. These remodeled nucleosome particles 'The Remosomes' possess characteristic structural features. Our AFM studies show that ~180 bp of DNA is associated with the histone octamer as compared to ~147 bp in the canonical nucleosomes. Using DNaseI footprinting and EC-M we have shown that the path of DNA around the histone octamer is highly perturbed. Moreover, these particles represent an ensemble many different structures rather than one defined specie. The novel 'in gel one pot assay' showed that accessibility profile of these particles is completely different from that of canonical nucleosomes and they are accessible all along the path of DNA.

We have also addressed the question of inhibition of nucleosome mobilization due to incorporation of histone variant H2A.Bbd in the nucleosomes. We show that the docking domain of histone H2A is essential for SWI/SNF and RSC induced nucleosome sliding. Furthermore, we demonstrate that the reason for inability of these nucleosomes to slide is due to a faulty generation of 'Remosome' intermediates.

C. Keywords

a.   Mots clés en Français

Variants d'histones, remodelage de la chromatine, nucléosome, RSC, SWI SNF, chromatine, H2A.Bbd

b.   Keywords in English

Histone variants, chromatin remodeling, nucleosome, RSC, SWI/SNF, chromatin, H2A.Bbd

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List of Abbreviations

ACF	ATP-dependent Chromatin assembly and remodeling Factor
AFM	Atomic Force Microscopy
ATP	Adenosine-5'-triphosphate
Bbd	Barr Body Deficient
bp	Base Pair
CHD	Chromodomain Helicase DNA-binding
ChIP	Chromatin Immuno Precipitation
CHRAC	CHRomatin Accessibility Complex
Da	Dalton (1g/mol)
dd	Docking Domain
DNA	Deoxyribo Nucleic Acid
DTT	Dithiothreitol
ECM	Electron Cryo Microscopy
EDTA	Ethylene Diamine Tetra Acetic acid
EM	Electron Microscopy
EMSA	Electrophoretic Mobility Shift Assay
HA	Hemagglutinin
ISWI	Imititation SWItch
Lc	Length of DNA complexed within the nucleosome
NaCl	Sodium Chloride
NCP	Nucleosome Core Particle
NFR	Nucleosome Free Region
NURF	Nucleosome Remodeling Factor
ORF	Open Reading Frame
PAGE	Polyacrylamide Gel Electrophoresis
PCR	Polymerase Chain Reaction
RSC	Remodels Structure of Chromatin
SDS	Sodium Dodecyl Sulfate
ΔL	Distance of nucelosome from the center of the fragment

Chapter I: Introduction

Chromatin Structure, Organization and Dynamics

The genetic instructions which are used for development and functioning of all living organisms are contained in nucleic acid called as deoxyribonucleic acid (DNA). DNA contains instructions for synthesis of other components of cells. Other DNA sequences between the genes have structural purposes and are known to be involved in regulation of gene expression. DNA was first isolated in 1869 by Friedrich Miescher as a microscopic substance in the pus of discarded surgical bandages. Since, it resided in the nuclei of cells, he called "nuclein" (Dahm R, 2005). Later, in 1919 Phoebus Levene identified the base, sugar and phosphate components of nucleotides (Leven P, 1919) and suggested that DNA consisted of a string of nucleotides linked together through the phosphate groups. Finally, DNA's role in heredity was confirmed in 1952 by the famous Hershey-Chase experiment (Hershey and Chase 1952) and based on X-ray diffraction data by Rosalind Franklin and the information that the bases were paired; James D. Watson and Francis Crick suggested the double helix structure of DNA what is now accepted as the first accurate model of DNA structure (Watson and Crick, 1953).

In the nucleus DNA exists as a complex structure called chromatin, a combination of DNA with proteins. The term 'Chromatin' was suggested for the first time by W. Flemming (~1880), owing to its affinity to stains, while studying the process of nuclear division. The purpose of chromatin organization is to package DNA into a smaller volume to fit in the cell, to strengthen the DNA to allow mitosis and meiosis, and to serve as a mechanism to control vital processes like transcription, repair and DNA replication. Three basic levels of chromatin organization occur in the cell:

i.   DNA wrapping around nucleosomes - leading to the primary structure of chromatin called as the "beads on a string" structure.
ii. A 30 nm condensed chromatin fiber resulting from specific interactions between nucleosomes (Secondary structure of chromatin).
iii. Highest level of DNA packaging resulting in the most compact form of chromatin: the metaphase chromosomes.

I.1 The Nucleosome

The basic repeat elements of chromatin are the nucleosomes which are interconnected by stretchess of linker DNA. Kornberg (1974) first defined nucleosomes to be composed of about 200 bp DNA associated with two copies each of the core histones H2A, H2B, H3 and H4. Hence, the protein core of nucleosomes is also called as histone octamer. Further, nucleosomes an also be associated with one unit of linker histones. Non-condensed nucleosomes without the linker histones resemble "beads on a string of DNA" under an electron microscope (Figure I.1, Thoma et al 1979; Olins and Olins 1974). Linker histones such as H1 or H5 and their isoforms are involved in chromatin compaction and bind to the linker region of the DNA at the base of the nucleosome near the DNA entry and exit site (Zhou et al., 1998). The structure of nucleosome is highly preserved in all eukaryotes due to various antagonistic selective pressures during evolution. The first is the need for compaction of DNA. Indeed, in a human cell the DNA, about 2 meter long in extended form, has to be compacted to fit within the nucleus about 10 μm in diameter. On the other hand the cell must be able to access specific regions in its genome in order to produce certain RNA (transcription) or to duplicate its contents (replication) or to repair damage to its DNA. These vital needs for the cells led to a structure compact and stable but quickly modifiable and very dynamic at the same time.
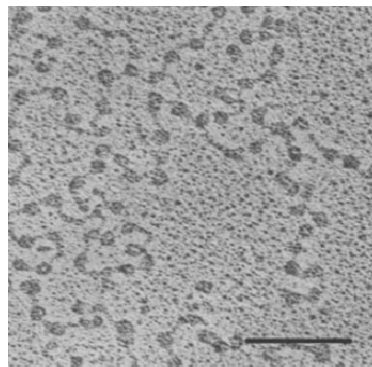
Figure I.1 Nucleosomes: Beads-on-a-string model. Electron microscopic image of H1-depleted isolated chromatin. Adapted from Thoma et al., 1979

I.1.1 Histones

Histone were discovered as an acid extractable material isolated from avian erythrocyte nuclei and first described by A. Kossel (1884) which he termed as 'histon' (Olins and Olins, 2003). Histones are small basic proteins of about 10-15 kDa (100 to 130 amino-acids) found in all

eukaryotes and are among proteins that are most conserved during evolution. Histone sequences have even been identified in many archaeal genomes and they constitute a family of proteins that are structural homologs of the eukaryotic core histones and are called as archaeal histones (Sandman and Reeve, 2006). There are 5 canonical forms of histones: ·

H2A (14 kDa), H2B (14 kDa), H3 (15 kDa) and H4 (11 kDa) are called as core histones and H1 (21 kDa) is called as linker histone. The core histones have three functional domains:

(i) Histone fold domain,

(ii) N-terminal tail domain, and

(iii) Various accessory helices and less structured regions.
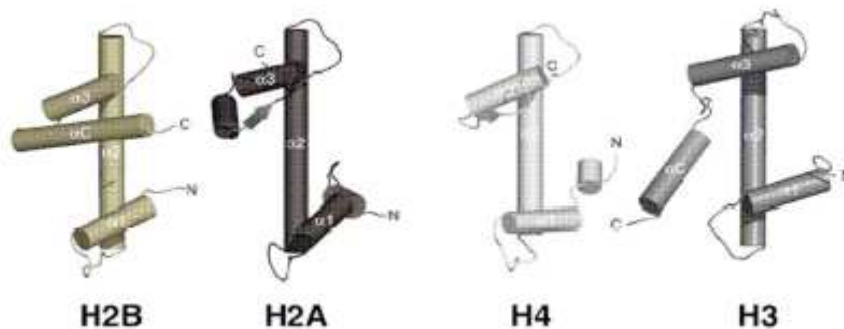
Figure I.2 Histone fold of the core histones (H2A, H2B, H3 and H4). All adopt the same secondary structure called as "histone fold", which consists of a sequence of three propellers α represented by the cylinders. The histone fold is present at the base of histone dimerisation (H2A-H2B) and (H3-H4). Adapted from Sondermann et al., 2003

The "histone fold" is composed of symmetric duplication of helix-loop-helix motif with a long median helix and two shorter terminal helices joined by loops to the median helix (FigureI.2) allowing the histones to interact between them (H2A-H2B) and (H3-H4), via hydrophobic interactions (Sondermann et al., 2003). These heterodimeric pairing is commonly called as "handshake" pairing wherein median helices of the partners align in opposite orientations (Arents et al., 1991). In the absence of DNA and under conditions of moderate salt concentration (150 mM NaCl), H3 and H4 join to form a tetramer $(H3-H4)_2$ whereas H2A and H2B remain associated in the form of dimer (Figure I.3). In high salt

concentration (2M NaCl), the octamer of histones forms spontaneously, *in vitro* (Eickbush and Moudrianakis, 1978).

Besides histone fold, each histone has distinct N-terminal and C-terminal regions. N-terminal tails of histones are located outside of nucleosomes and are subjected to covalent modifications which may lead to modification of local chromatin structure either directly or through other interacting proteins. These accessible regions serve as a platform for interaction between chromatin and regulatory proteins. The amino-terminal parts of the histones do not take part significantly in the structure of the nucleosome; they seem to be rather committed in interactions with other proteins or others nucleosomes. Tails of the histones H2B and H4 in particular are important for the formation of higher order structure of chromatin. The integrity of the tail of H4 is necessary for the formation of 30 nm fiber (Dorigo et al., 2003) and the amino-terminal part of H2B is necessary for the chromosome assembly (de la Barre et al., 2000; de la Barre et al., 2001). This higher order architecture is facilitated and stabilized by linker histones.
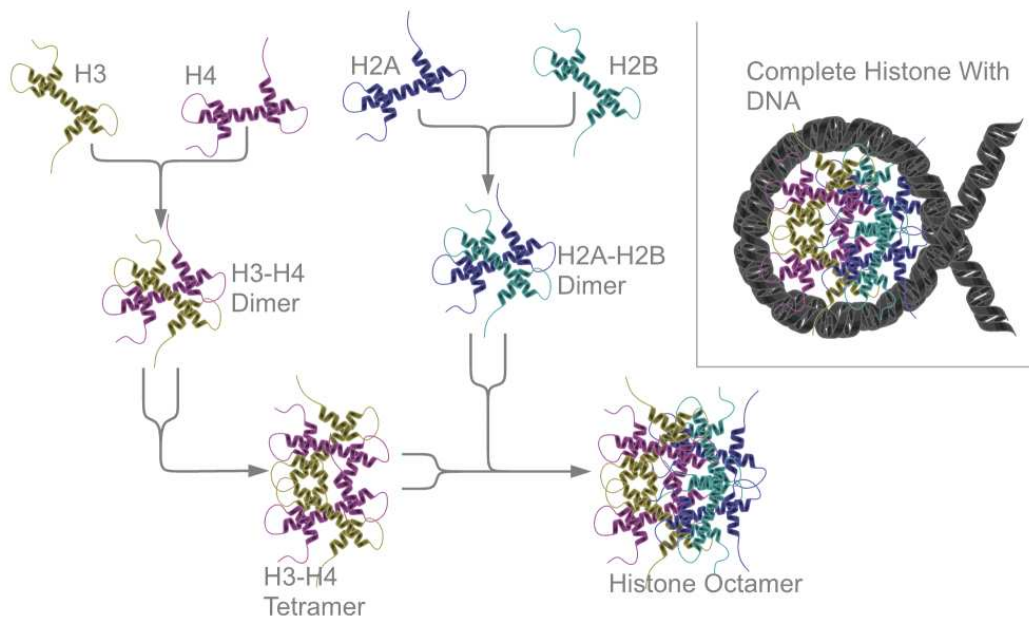
Figure I.3 Nucleosome assembly. The four core histones are organized in a tetramer $(H3-H4)_2$ and two dimers (H2A-H2B). Under ionic concentrations lower than 0.5 M and in the presence of DNA these species are assembled to form the nucleosome or "nucleosomal core particle" (NCP). Adapted from Richard Wheeler.

The linker histone H1 represents another family of histones as it does not have the same structure as the core histones. It adopts a tripartite structure, made up of a conserved globular central domain of about 80 amino acids flanked by long, highly positive N- and C-terminal tails which diverge by their size and their sequence among different H1 variants (Wolffe et al., 1997). The globular domain of H1 interacts with the nucleosome core particles at the entry and exit site of DNA into the core particle. It has been shown to influence the angle of entry/exit of linker DNA and many have suggested its role in organization of 30 nm fiber. However, knockout studies of H1 have posed question on its significance for nuclear assembly. Moreover for the location earlier it was thought to be present at the nucleosomal dyad axis (Widom, 1989) but Zhou et al., (1998) argued it to be positioned asymmetrically, compared to the centre of symmetry of the nucleosome. The debate on its actual position and function is still on (Figure I.4, Brown et al., 2006).
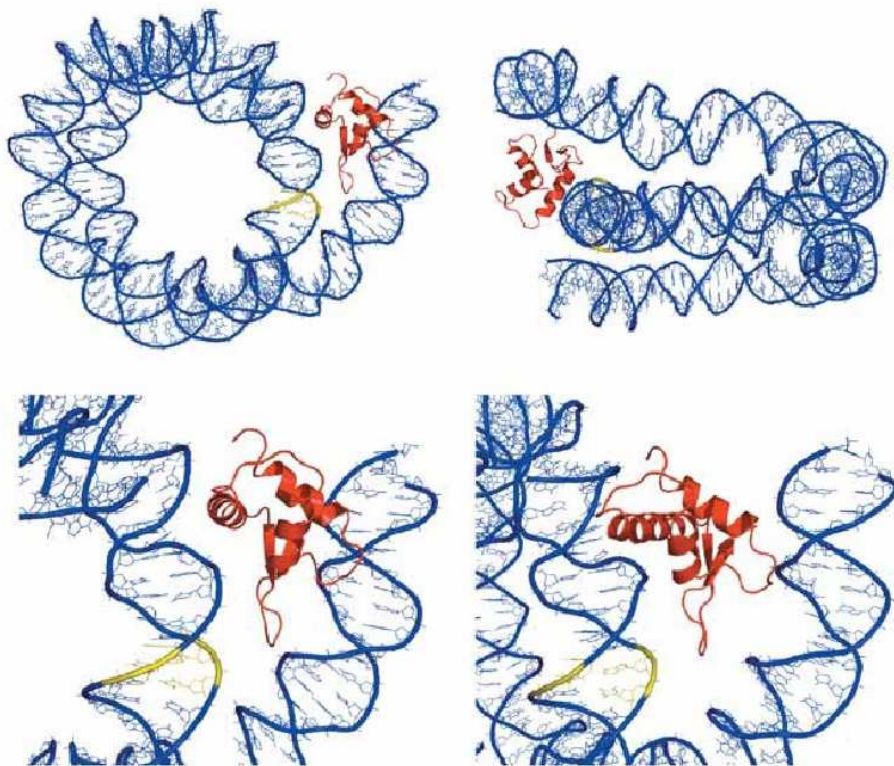
Figure I.4 Structure and potential position of linker histone on nucleosome. Linker histone is represented in red and nucleosomal dyad in the blue DNA is represented in yellow. Adapted from Brown et al., 2006.

I.1.2 Crystal structure of the nucleosome

The structure of histone octamer in absence of DNA was solved by X-ray crystallography at 3.1 Å resolution (Arents et al., 1991) and later the crystal structure of complete nucleosome core particle was solved at 2.8 Å resolution (Luger et al., 1997) providing details of protein and DNA interactions within the nucleosome. In the histone core, H2A and H2B form two dimers (H2A-H2B) whereas H3 and H4 are present in the form of a tetramer $(H3-H4)_2$. Structurally, the two dimers (H2A-H2B) enclose tetramer $(H3-H4)_2$ and form a sandwich structure around which 147 bp DNA is wrapped in about 1 ¾ left superhelical turns (Figure I.5). The nucleosome dimensions derived from this structure are 11 nm in diameter and 6 nm in height (Luger et al., 1997). The nucleosome displays an apparent two-fold symmetry with the axis passing through the octamer and intersects DNA perpendicularly at midpoint of the wrapped sequence. DNA interacts with the histone proteins through 14 hydrogen bonds at every 10 bp length. This bonding makes nucleosomes electrostatically stable between 20 and 30 kT according to the ionic conditions, temperature and sequence (Richmond and Davey, 2003).
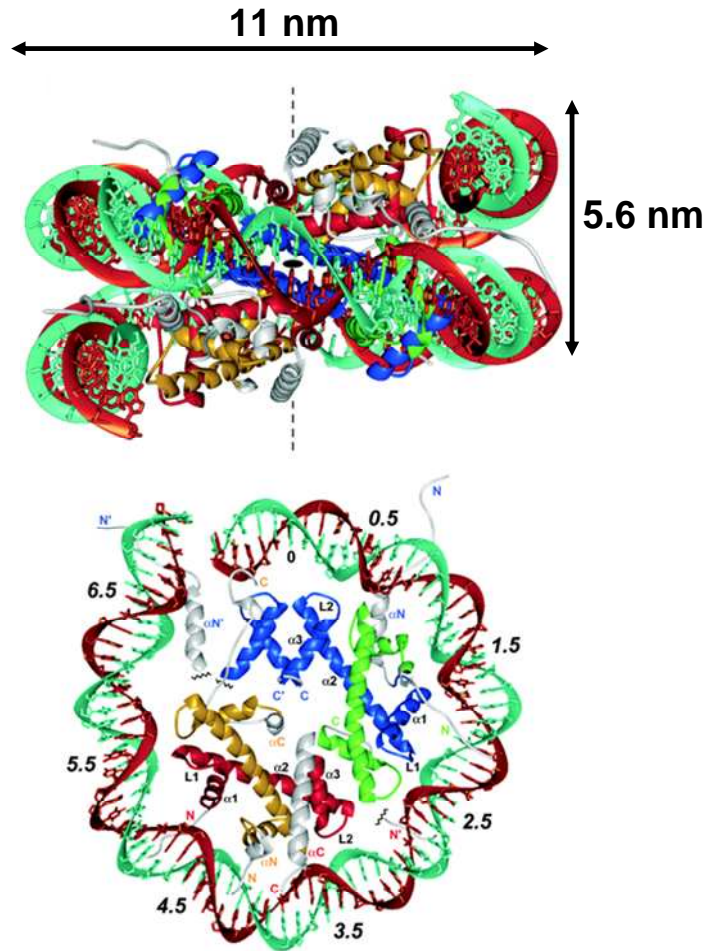
Figure I.5. Structure of the 147 bp nucleosome core particle at 1.9 Å resolution. (a) View down the axis of 2-fold pseudo-symmetry (dyad axis, black) with the DNA superhelix axis oriented vertically (broken line). The dyad axis bisects the central base-pair. The 147 bp palindromic DNA sequence shows nearly perfect 2-fold symmetry relating the two 73 bp halves of the DNA superhelix extending from the central base-pair. The DNA strands are cyan and brown. The histone-fold domains of the histone proteins are blue for H3, green for H4, yellow for H2A and red for H2B. The histone-fold extensions and N-terminal tail regions shown are white. (b) View down the DNA superhelix axis showing one half of the structure to illustrate the organization of histone and DNA. Colors are as for (a). The superhelix locations are labeled at the DNA-binding sites of the histone-fold pairs and the H3-αN helix (SHL: 0.5, 1.5, 2.5, 3.5, 4.5, 5.5, 6.5). The central base-pair is indicated (0). The histone-fold substructure for histones H3 and H2B are labeled (α1, L1, α2, L2, α3) as are histone-fold extensions (αN, αC) and segments of the N and C-terminal tails (N, C, N′, C′). Adapted from Davey et al., 2002.

I.2 Higher orders of Chromatin structure

In the chromatin, 11nm fiber of nucleosomal beads on DNA string compacts to form higher levels of organization. Between the final structure of chromatin i.e. the mitotic chromosome and the nucleosomal array, certain intermediate levels of organization have been postulated (Figure I.6).
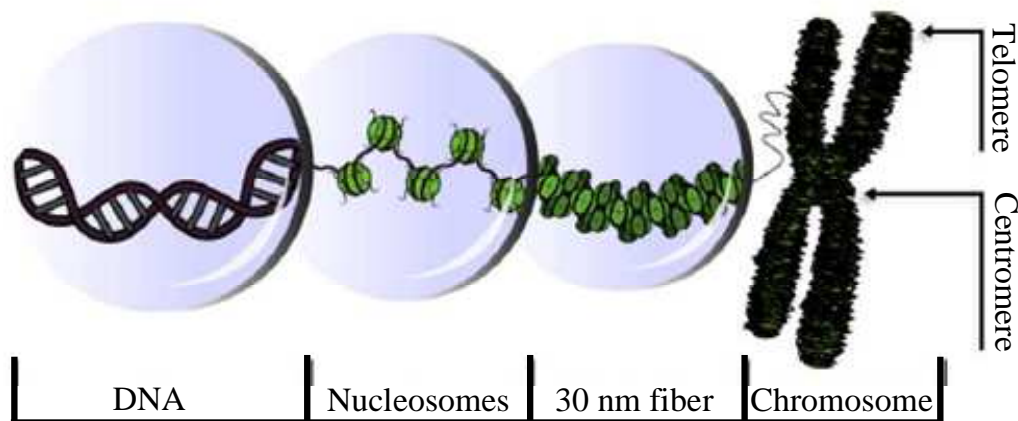
Figure I.6 Different orders of chromatin architecture. The DNA is wrapped around the histone octamers to fom nucleosomes which are connected by linker DNA. This represents the primary structure of chromatin. The compaction of this array of nucleosomes constitutes the secondary structure of the chromatin, commonly called as 30 nm fiber. The extreme compaction of chromatin is illustrated by the mitotic chromosome. The mitotic chromosome consists of four arms protected by a telomeric end. The point where anchoring of the mitotic spindle occurs is named as centromere. Adapted from Boulard 2007.

Under physiological conditions the 11 nm fiber further compacts and forms 30 nm chromatin fibers which subsequently folds into higher order structures. Indeed, preliminary studies on chromatin, which were carried out by employing electron microscopy and digestion with nucleases, revealed the presence of a regular fiber which compacted in the presence of linker histones (H1 or H5) and by interactions between H2A with the N-terminal of histone H4. However, since then, the general information and the internal organization of this type of fiber are largely prone to debate and several contradictory models have been proposed. Two principal architectures of 30 nm fiber arrangement proposed are the *solenoid* and *zig-zag* *models* (Figure I.7).
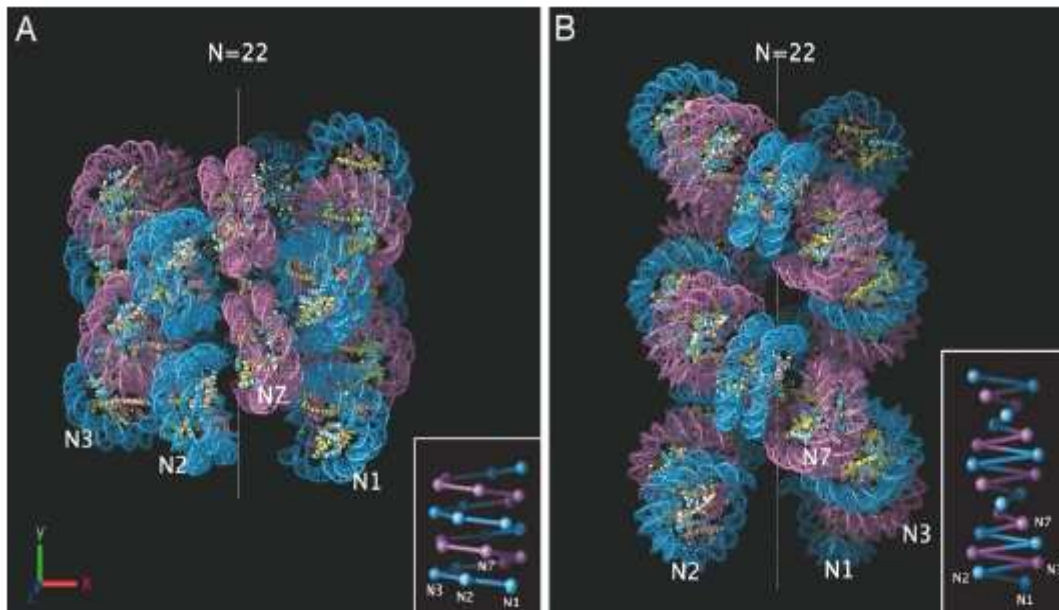
Figure I.7 Models of 30 nm fiber organization (A) Solenoid Model, (B) Zigzag model. Adapted from Robinson et al., 2006.

Solenoid or single-start helices involve wrapping of a tightly packed array of nucleosomes into a helix, which is stabilized by inter-nucleosomal interactions. In order to overcome the constraint posed by linker DNA and to establish necessary contacts between successive nucleosomes the linker DNA should be bent inside the fiber or must continue the superhelical path of nucleosomal DNA between nucleosomes (Finch and Klug, 1976). 'Zig-zag' model proposes existence of two helices connected by straight linker DNA (Bednar et al., 1998). Here, consecutive nucleosomes are alternatively packed and dimensions of the helix depend on linker length which is not true for nucleosome dependent solenoid model. Till date the issue of two-start versus one-start helices is not settled. Schalch et al (2005) described X-ray crystallographic structure of reconstituted tetranucleosome wherein very short linker DNA connects two stacks of non-consecutive nucleosomes thus supporting 'zig-zag' model. On the other hand, Robinson et al. (2006) combined the techniques of chromatin reconstitution and electron microscopy and found that helix diameter and length remains almost constant over considerable linker length variations as expected on the basis of solenoid model. Recently, van Holde and Zlatanova (2007) have reviewed the 30 nm fiber structures and discussed the controversy associated with it from last 30 years.

Internal organization of this fiber depends on many parameters like concentration of divalent ions and spacing between consecutive nucleosomes for example, the diameter of the fiber can

vary from 30 to 40 nm when space between nucleosomes is changed from 50 to 70 bp (Robinson et al., 2006). Hence, the organization of nucleosomes within chromatin is highly dynamic and changes according to the particular conditions in the microenvironment like the presence of transcription factor or the regularity of the positioning of the nucleosomes. Chromatin organization is stabilized by multiple chromatin-associated proteins especially the linker histones. Linker histones are located between two nucleosomes and stabilize both intramolecular folding as well as fiber-fiber interactions (Carruthers et al., 1998). Furthermore, histone tails of core histones interact with other proteins to stabilize the nucleosomal organization (Hansen, 2002; Zheng and Hayes, 2003). These fibers fold further to form compacted tertiary structures but its detailed structure is still not well understood, it has been postulated that the fibers roll-up on itself to form thicker fibers, called as chromoneme model (Belmont and Bruce, 1994). These structures then interact with nuclear matrix to form more condensed and organized sections. Various experimental observations tend to show the presence of loops (from 200 to 300 nm diameter is several hundreds of kbp) or chromomers (Cook and Brazell, 1975; Old et al., 1977; Fraser and Bickmore, 2007). One functional model proposed by Cook (1995) is that each one of these loops could constitute a unit of transcription. These fibers finally fold into an organized manner to form a chromosome (Fisher and Merkenschlanger, 2002; Hancock 2000). Compact organizations within chromosomes probably correspond to nonactive zones of chromatin that are not transcribed by the cell (Dubochet et al., 1988; Woodcock 1994; Gilbert and Allain, 2001) and are called as 'heterochromatin'. In the active and/or less dense zones of genes, nucleosomes are less regularly organized and chromatin might be present in a conformation of 11 nm fiber (McDowall et al., 1986; Horowitz et al., 1994; Gilbert et al., 2005). This poses a question on the existence of 30 nm fiber *in vivo*.

I.3 Chromatin Territories

In the nucleus chromatin is organized as condensed regions with a defined spatial arrangement so that distinct compartments within the nucleus can influence chromatin dynamics such as gene expression and silencing (Pederson, 2004; Baxter et al., 2002; Chubb and Bickmore, 2003). This positioning of chromatin within nucleus is called as 'chromatin territories' and was first described by Heitz in 1928 as less stained, decondensed 'euchromatin' and more compact, highly stained 'heterochromatin' (Passarge 1979). Euchromatin contains highly accessible DNA and most protein coding genes are located

within this region. It decondenses during interphase and is replicated early in S-phase. On the other hand, heterochromatin are transcriptionally inactive regions and replicated late in the cell cycle (Fisher and Merkenschlager, 2002; Grewal and Elgin 2002; Grewal and Moazed, 2003). These regions are mainly associated with centromeres and telomeres of chromosomes, however short stretches of interspersed heterochromatin are also found throughout the chromosome (Fahrner and Baylin, 2003). It is thought to stabilize the genome and regulate gene expression during development and differentiation (Grewal and Moazed, 2003). Sometimes heterochromatin can spread to euchromatic regions leading to changes in their chromatin structure thus resulting in gene inactivation (Reuter and Spierer, 1992; Schotta et al., 2003). Furthermore, histone modifications and special histone variants are known to be associated with heterochromatin. Establishment and maintenance of heterochromatic state of chromatin is mainly achieved by chromatin remodeling by histone modification, DNA methylation and RNAi machinery (Vermaak et al 2003).
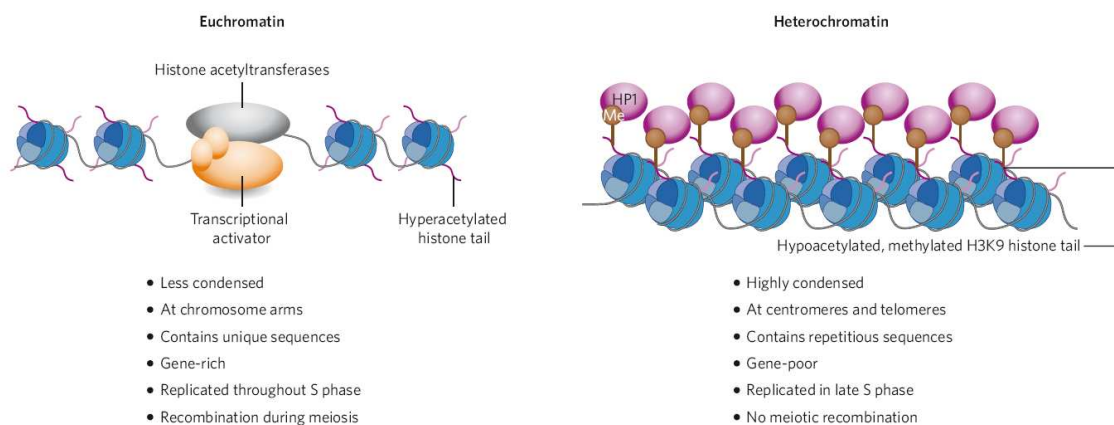
Figure I.8 General properties of euchromatin and heterochromatin. Adapted from Grewal and Elgin 2002.

I.4 Regulation of Chromatin Dynamics

Nucleosomes, as shown by the crystal structure, exhibit strong interaction between DNA and core histones and are highly stable but flexible structures. Chromatin, at all levels of organization, is very dynamic and this plasticity is crucial to ensure proper functioning of the cell. Modification of chromatin structure is the prime step in regulation of all the processes for which genetic information is stored in the DNA. Indeed, chromatin provides the substrate

upon which most important biological processes like transcription, replication, repair and recombination takes place. These processes require quick changes in chromatin organization and structure. In order to make the DNA accessible to enzymatic machinery, the compacted DNA fiber needs to be unraveled (van Holde and Zlatanova, 1996). Physical parameters such as the affinity of the DNA sequence to the histone octamer or the intrinsic curvature of the DNA sequence can have strong effects on the structure of chromatin. Indeed, nucleosomes on some DNA sequences are more prone to temperature induced octamer repositioning than sequences which have more affinity towards the octamer (Beard et al., 1978; Meersseman et al., 1992; Falus et al., 2004; Lowary and Widom, 1998). Moreover, nucleosomes are able to adapt to strong distortions induced by binding of ligand on the DNA without losing the contact with the histone octamer (Edayathumangalam and Luger, 2005). Certain transcription factors such as NF-κB can bind to DNA without inhibition or major modification of the nucleosome (Angelov et al., 2004). This intrinsic dynamics of the nucleosome (or breathing) does not allow the complete DNA to be accessible for all the cellular machineries. Moreover this "breathing" of the nucleosomal DNA is limited to the ends of the nucleosome (Anderson et al., 2002). Therefore, cells have developed certain mechanisms to ensure modulation of DNA accessibility. Three principal methods to ensure this plasticity are, as described in the following sections, incorporation of histone variants, histone covalent modifications and ATP dependent chromatin remodeling.

I.4.1 Incorporation of Histone variants

The structure of chromatin can be adapted to perform specialized functions by variation in its core histone composition. Histones deposited at the time of DNA replication are called as conventional histones (H2A, H2B, H3 and H4). They represent majority of histones (60-90 %) in the cells and are synthesized only during S-phase of cell cycle. However, synthesis of histones out of this phase of replication also takes place. These are non-allelic counterparts of conventional histones and are called as 'variants'. They can be deposited in the nucleosome a manner independent of the replication and have the capacity to substitute canonical histones within the nucleosome. Hence, these are also called as 'replacement histones'. Except H4, multiple variant forms of all other core histones exist, however, alternative mRNA forms of H4 also seem to be present (Boulard et al., 2007; Poirier et al., 2006; Gendron et al., 1998). The percentage identity of each histone with its conventional counterpart is highly variable (from 48 to 99.9%) (Figure I.9). Some are much conserved and are present throughout the

animal kingdom such as H2A.Z whereas some are present only in the mammals, like H2A.Bbd. The presence of histone variants within the nucleosome modifies the structure and dynamics of the nucleosomes leading to significant impact on several cellular processes involving DNA, including transcription, repair, cell division and meiosis; and could have important epigenetic consequences as well. Contrary to their conventional counterparts, mRNA of variants is deprived of stem loop structure at its 3' end, which is necessary for the degradation controlled by the cellular cycle (Pandey et al., 1987). In place of this stem loop structure, these mRNA's are polyadenylated, which increases their stability (Challoner et al., 1989). These specificities imply that histone variants are incorporated in the nucleosome by various ways and are dealt with by specific protein chaperones.
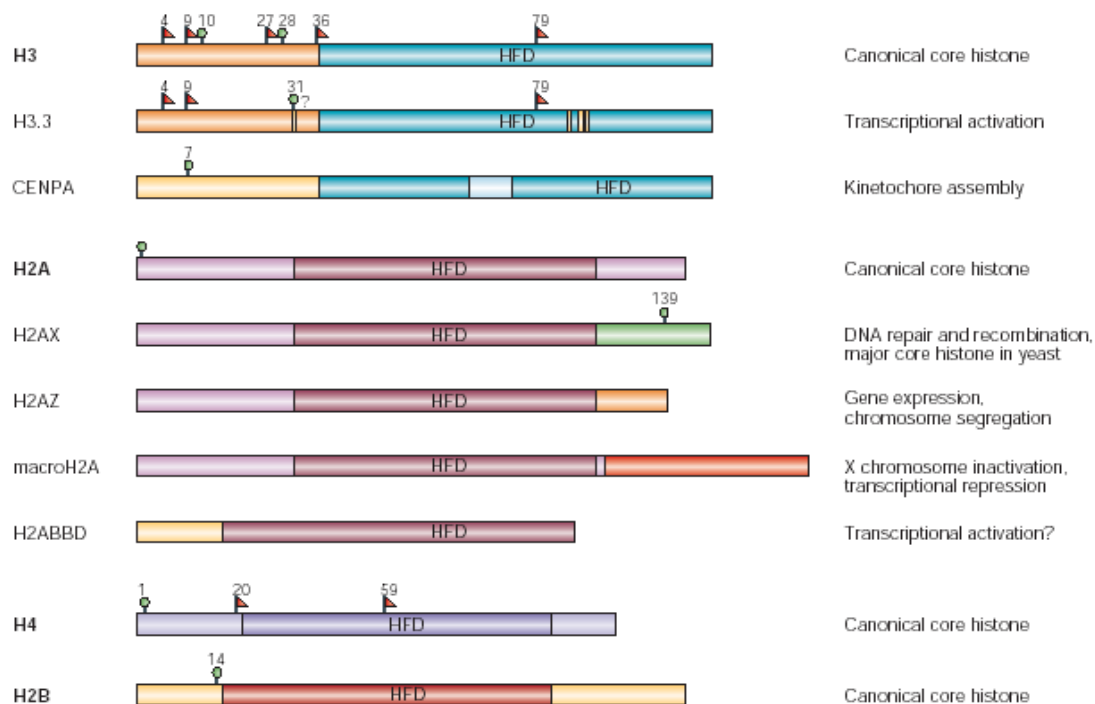
Figure I.9 Canonical histones and their variants. Adapted from Sarma and Reinberg, 2005.

I.4.1.1 H3 variants

In mammals there are five isoforms of H3: H3.1, H3.2, H3.3, H3t and CENP-A. There are minor differences between the variants, but a very strong positive selection is observed upon each histone (Marzluff et al., 2002). Each difference, even small, thus seems to be related to an important functional consequence.

Centromere specific H3 variant (CenH3 or CENP-A) is absolutely required for assembly of the proteinaceous kinetochore to which spindle microtubles attach during cell division (Blower and Karpen, 2001). Inactivation of this variant of histone is lethal at the embryonic stage in the mouse, as it prevents correct mitosis. The assembly of CenH3-containing nucleosome is independent of replication (Ahmad and Henikoff, 2001; Shelby et al., 2000). In contrast to the nearly invariant N-terminal tail of canonical H3, the N-terminal tail of CenH3 is highly diverse and significantly varies in length and sequence among different species (Malik and Henikoff, 2003).

Another H3 variant, H3.3 is very similar to H3 and differs only at 4 amino acid positions, three of which determine nucleosome assembly behavior. Changes from H3 to H3.3 form allow replication independent assembly (Ahmad and Henikoff, 2002). It is enriched in the transcriptionally active zones of chromatin of insects, plants and humans (Ahmad and Henikoff, 2002; McKittrick et al., 2004; Chow et al 2005). H3.3 containing complexes are copurified with replication-independent histone chaperone, HIRA, which differentiates it from other H3 which gets copurified with CAF-1 (replication competent assembly complex) (Tagami et al., 2004). Furthermore, this variant is post-translationally modified in a way that favors transcriptional activity, namely hyper-acetylation and dimethylation of H3K36 and H3K79 (Hake and Allis, 2006).

The variants H3.1 and H3.2 were confused with each other for a long time, but the study of their post translational modifications suggest distinct roles (Hake and Allis, 2006). H3.2 is methylated on H3K27 and is implied in gene silencing, whereas H3.1 has marks associated with activation of genes (H3K14ac) as well as repression of transcription (H3K9me2).

I.4.1.2 H2A variants

Besides the conserved histone-fold domain, the histone H2A has very long N-terminal tail which intercalates between two turns of nucleosomal DNA and its C-terminal tail has a docking domain through which it can interact with $(H3-H4)_2$ tetramer via N-terminal tail of H4 (Luger et al., 1997; Suto et al., 2000). This interaction between H2A-H2B diamer with the $(H3-H4)_2$ tetramer is essential for compaction of chromatin fiber (Horn et al., 2002; Zhou et al., 2007). Being an important player in regulation of chromatin dynamics a number of variants of H2A exists. Based on the sequence these variants can be described by their evolutionary origin (Figure I.10, Malik and Henikoff, 2003). H2A.Z has originated very early

in eukaryotic evolution and is present in the mammals, birds (H2A.F), the *Drosophila* (H 2Av), *C. elegans*, sea urchins (H2AZ/F), various protozoans like *Tetrahymena* (H2Ahv1) and yeast (Htz1) (Raisner et al., 2005). Likewise, H2A.X is also present in all eukaryotes whereas the variant macroH2A exists only in vertebrates and H2A.Bbd is found exclusively in mammals (Malik and Henikoff, 2003; Eirin-Lopez et al., 2008).
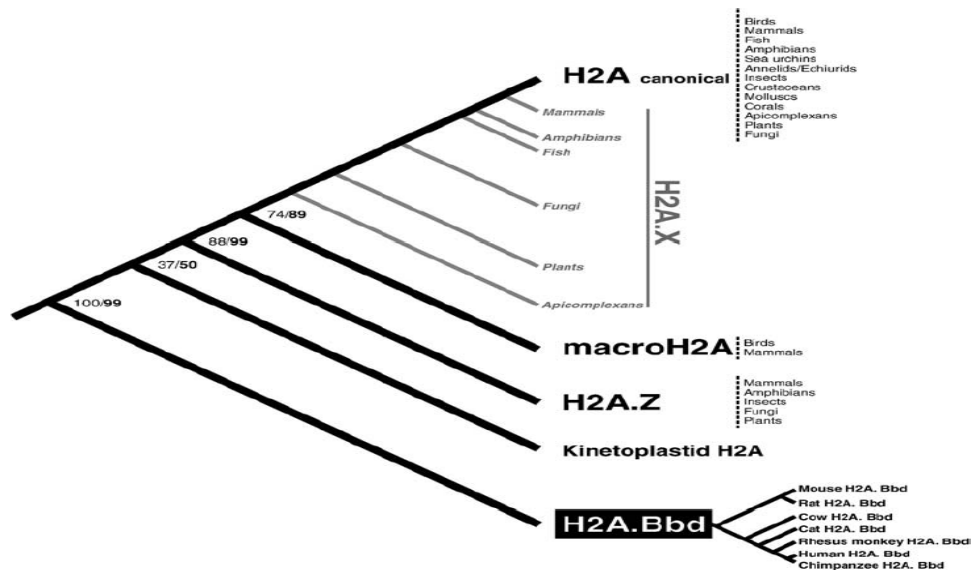
Figure I.10 Phylogenetic tree of H2A variants. Adapted from Gonzalez-Romero et al., 2008.

I.4.1.2.1 H2A.Z The variant H2A.Z shares only around 60% homology with the canonical H2A and 90% homology between species. The resolution of crystal structure of nucleosome containing H2A.Z by Suto et al., (2000) revealed that DNA trajectory is not distorted by replacement with this variant however, protein-protein interactions are affected. Differences in affinity between H2A-H2B dimer and the core tetramer were observed. Three hydrogen bonds were found to be lost, thus destabilizing the interaction between H3 and H2A.Z. However, the dimer H2A.Z-H2B forms a strong acidic patch and a divalent cation binding site on the surface through which it could bind more strongly to H4 tail or other interacting non-histone proteins (Suto et al., 2000). Also, it could support the formation of 30 nm fiber. Several studies reporting contradictory physical properties of H2A.Z variant have been published; these differences in observations are might be due to the difference in techniques used in the studies. For example, certain measurements conclude that the nucleosome containing H2A.Z would be less stable (Abbott et al., 2001) while others found it to be highly stable (Fan et al., 2002; Park et al., 2004; Jin and Felsenfeld, 2007).

H2A.Z has been observed to be located at yeast promoters and display a redundant role with ATP-dependent nucleosome remodeling complexes (Santisteban et al., 2000) and found to interact directly with transcriptional machinery during gene expression (Adam et al., 2001). Flaus et al., (2004) observed that H2A.Z nucleosomes can slide thermodynamically more quickly than conventional nucleosomes at around 30°C. This important observation strengthened their property similar to remodeling complexes such as SWI/SNF. Various groups determined genome wide localization of H2A.Z nucleosomes (Guillemette et al., 2005; Li et al., 2005a; Raisner et al., 2005; Zhang et al., 2005; Millar et al., 2006; Barski et al., 2007). All these works point towards localization of a large fraction of H2A.Z nucleosomes on promoter regions. However, the correlation between the presence of H2A.Z on the promoter and activity of the gene is not known for all and is still discussed. Recently, Baski et al., (2007) carried out high-resolution analysis of H2A.Z positioning in human genome, using SOLEXA© sequencing technique and found that, contrary to yeast, the presence of H2A.Z on the promoter is correlated with an active transcription in humans. These studies with high-resolution positioning of H2A.Z on the genome show that H2A.Z is strongly enriched in the nucleosome free area (NFR) of promoters (Raisner et al., 2005; Barski et al., 2007). This work shows that two nucleosomes containing H2A.Z flank the NFR of the promoter, in yeast and in human (Raisner et al., 2005; Barski et al., 2007). This led to identification of a 22 bp consensus sequence that could promote formation of NFR and incorporation of H2A.Z in both nucleosomes flanking this area (Raisner et al., 2005). In yeast, acetylation of histones also directs incorporation of H2A.Z in euchromatic regions (Raisner et al., 2005; Zhang et al., 2005). Hence, H2A.Z targets specific nucleosomes within promoters and can create promoter-specific chromatin structures, However, H2A.Z enrichment in active chromatin may even lead to repression of gene expression (Dhillon and Kamakaka, 2000) hence; its role in functional chromatin dynamics is enigmatic.

In vitro, the presence of H2A.Z facilitates nucleosomal fiber compaction, but inhibits oligomerization (Fan et al., 2002). This suggests that chromatin containing H2A.Z could be present in the heterochromatic region. In parallel, HP1α (a protein known to be related to constitution and compaction of heterochromatin) was found to bind preferentially to chromatins reconstituted with H2A.Z *in vitro* and absence of H2A.Z changes HP1α protein localization, *in vivo* (Fan et al., 2004). These results indicate involvement of H2A.Z in formation of pericentric heterochromatin.

H2A.Z is assembled by SWR1, a member of SWI/SNF family (Krogan et al., 2003; Kobor et al 2004; Krogan et al., 2004; Mizuguchi et al., 2004) but the exact mechanism is still not known. Another histone chaperone called Chz1 preferentially deposits H2A.Z-H2B dimer (Luk et al., 2007). Taken together, the available data suggests that H2A.Z plays important role in several cellular processes and can affect architecture of chromatin towards both increased gene expression as well as gene silencing. These distinct and even antagonistic functions are probably dependent on the particular dynamics of these nucleosomes and their distinct mechanisms of deposition.

I.4.1.2.2 H2A.X

H2A.X represents about 10-15% of total H2A in most of the mammalian cells. Its sequence is very similar to the canonical H2A at amino terminal and core regions however, varies considerably at carboxy-terminal end (West and Bonner, 1983). In humans, the carboxy-terminal end of H2A.X differs in both length as well as sequence from H2A. It contains 20 amino acids more than H2A and exhibits homology with lower vertebrate species. In particular, it contains a very conserved tetrapeptide motif (Ser-Gln-acidic-aliphatic) whose serine (position 139) gets phosphorylated upon introduction of a double strand break (Marzluff and Pandey, 1988; Rogakou et al., 1998; Li et al., 2005b). In mammals, a second (S,T)Q motif is present upstream of this region which also gets phosphorylated (position 136) but to a lesser extent (Rogakou et al., 2000). Furthermore, another upstream conserved region is formed by GKK cassette and posttranslational modifications of these three residues play important functional role (Li et al., 2005b).

Redon et al., (2002) demonstrated phosphorylation of H2A.X (γH2A.X) as a general phenomenon correlated with DNA double strand breaks and suggested its role in DNA repair. This phosphorylation is carried out by three kinases of PIKK family namely, ATM, DNA-PK and ATR (Stiff et al., 2004). ATM (ataxia telangiectasia (A-T) mutated protein) is a crucial kinase for the signal transduction DSB pathway (Savitsky et al., 1995) and is known to play a dominant role in H2A.X phosphorylation than the other two kinases (Burma et al., 2001; Fernandez-Capetillo et al., 2002; Redon et al., 2002). The phosphorylation of H2A.X could either directly open chromatin or can affect histone interactions and thus carryout opening of 30 nm fiber (Li et al., 2005b). For DNA repair chromatin decondensation is a prerequisite. The phosphorylated serine of γH2A.X is present near the C-terminal end and is accessible for

interaction with other proteins. This interacts with and plays essential role in accumulation of various DNA repair proteins and formation of DNA damage-induced repair foci. However, Celeste et al., (2003) demonstrated that it does not signal migration of repairing proteins to the damage site. γH2A.X is recognized by Arp4 (a common subunit of NuA4, Ino80 and Swr1 chromatin remodelers) as a mark of DNA damage (Downs et al., 2004). These complexes would then make it possible to modify structure of chromatin, so that the repair of the DNA can take place.

I.4.1.2.3 MacroH2A

MacroH2A (mH2A) is about three times more than the size of conventional H2A and is unique among the known histone variants with special tripartite structure. The N-terminal third of its amino acid sequence (amino acids [aa] 1 through 122) is 64% identical to major H2A. A C-terminal nonhistone region (aa 161 through 371) is linked to the histone homology domain via a linker region (aa 123 through 160) called as L1 loop. The large C-terminal region is also called as macro domain (Pehrson and Fried, 1992). In humans, two isoalleclic forms of mH2A, mH2A1 and mH2A2, are found and they exhibit about 80% homology (Chadwick and Willard, 2001; Costanzi and Pehrson, 2001). mH2A1 has two spliced variants, mH2A1.1 and mH2A1.2 (Chadwick et al., 2001).

The variant mH2A is associated with strong repression of transcription and is found to be especially enriched in inactive X chromosome (Costanzi and Pehrson, 1998; Costanzi and Pehrson, 2001). Moreover, it is also suggested to be involved in assembly and maintenance of heterochromatin (Chadwick and Willard, 2002; Choo et al., 2006; Costanzi and Pehrson, 1998; Grigoryev et al., 2004; Ma et al., 2005; Perche et al., 2000; Rasmussen et al., 2000). In senescent cells the silent senescence-associated heterochromatin foci (SAHF) were found to be enriched in mH2A (Zhang et al., 2005). mH2a can repress transcription at two levels. It can block posttranslational modifications of histones by blocking HAT p300 (Doyen et al., 2006a) and can interact with HDAC1 and HDAC2 (Chakravarthy et al., 2005) thus affecting acetylation status of mH2A containing and neighboring histones. Moreover, mH2A can block the action of chromatin remodelers through its L1 loop (Doyen et al., 2006a).

The crystal structure of only the macro domain has been resolved by several groups (Allen et al., 2003; Chakravarthy et al., 2005; Kustatscher et al., 2005) revealing its interesting structural and functional properties. It is characterized by a mixed alpha/beta fold and

exhibited similarity to the N-terminal binding domain of the *E. Coli* leucine aminopeptidase PepA and to members of the P-loop family of nucleotide hydrolysases (Allen et al., 2003). Recently, an ADP ribose binding motif has been found in the macro domain. Karras et al., (2005) demonstrated that the macrodomain contains a conserved pocket, which binds to ADP-ribose with high affinity. However, macro domain of only mH2A1.1 but not of mH2A.2 can bind with O-acetyl-ADP-ribose (Kustatscher et al., 2005). Since the two proteins differ only by a short amino acid stretch embedded within the macro domain, this points to a regulation of the binding of NAD (nicotinamide adenine dinucleotide) metabolites through alternative splicing (Kustatscher et al., 2005). Experimental data supporting this suggestion are still missing.

I.4.1.2.4 H2A.Bbd

H2A.Bbd (Barr body deficient) is the most recent discovered and least studied histone variant of the H2A family. It was found excluded from the inactive X chromosome, its name is thus derived from this localization property (Chadwick and Willard, 2001). It exhibits only 48% identity with the conventional H2A and is found to exhibit variations between species (Eirin-Lopez et al., 2008; Gonzalez-Romero et al., 2008). In contrast to mH2A, H2A.Bbd is shorter than H2A (115 amino-acids only) and lacks the flexible C-terminus and the histone H3 docking domain (Figure I.11, Chadwick and Willard, 2001; Luger et al., 1997). However, it contains a row of six arginines at its N-terminal tail, indicating that it could interact more strongly than the N-tail of H2A with nucleosomal DNA (Chadwick and Willard, 2001). Moreover, microccocal nuclease digestion of H2A.Bbd nucleosome suggests that only 118 bp are protected from the enzyme inside these nucleosomes (Bao et al., 2004) whereas the length of DNA protected by a conventional nucleosome is 146 bp. Recently, Doyen et al., (2006b) carried out a more limited digestion of the variable nucleosomes H2A.Bbd and found 130 bp to be organized around the H2A.Bbd octamer (Doyen et al., 2006b). Hence, nucleosomes containing H2A.Bbd instead of conventional H2A exhibit altered structure and chromatin remodelers like SWI/SNF and ACF are unable to remodel them (Angelov et al., 2004). Ultracentrifugation of H2A.Bbd nucleosomal arrays shows that compaction of these fibers is lower than that of conventional nucleosomes (Zhou et al., 2007) and addition of Mg2+ ions does not results in compaction of these fibers. Very few data are available as for localization of H2A.Bbd on the genome. Its biological role is not known. In humans, H2A.Bbd is detected by northern-blot in the testis and by RT-PCR in certain cellular lines (Chadwick et al., 2001).

Recently H2A.Bbd was also detected in brain, liver, kidney and prostate of mouse (Eirin-Lopez et al., 2008). *In vivo*, H2A.Bbd colocalizes with hyperacetylated regions suggesting its positive role in gene transcription (Chadwick et al., 2001). This assumption is supported by *in vitro* experiments showing that a array of H2A.Bbd containing nucleosomes is more easily transcribed and the histones are more effectively acetylated (Angelov et al., 2004). Measurements of FRAP, FRET and of sedimentation, highlighted that H2A.Bbd nucleosome is less stable than canonical nucleosome, which means that the H2A.Bbd-H2B dimer can be ejected and can be transferred more easily than H2A-H2B dimer (Angelov et al., 2004; Bao et al., 2004; Gautier et al., 2004). The instability of H2A.Bbd-H2B dimer implies that H2A.Bbd containing nucleosome has a more open structure facilitating access to effector proteins like HATs, transcription factors and the polymerase; this could explain the positive role of H2A.Bbd on the transcription. Due to greater accessibility, the DNA of H2A.Bbd nucleosome is more permissive for basic excision repair (BER) for which the canonical nucleosome posses a strong barrier (Menoni et al., 2007).
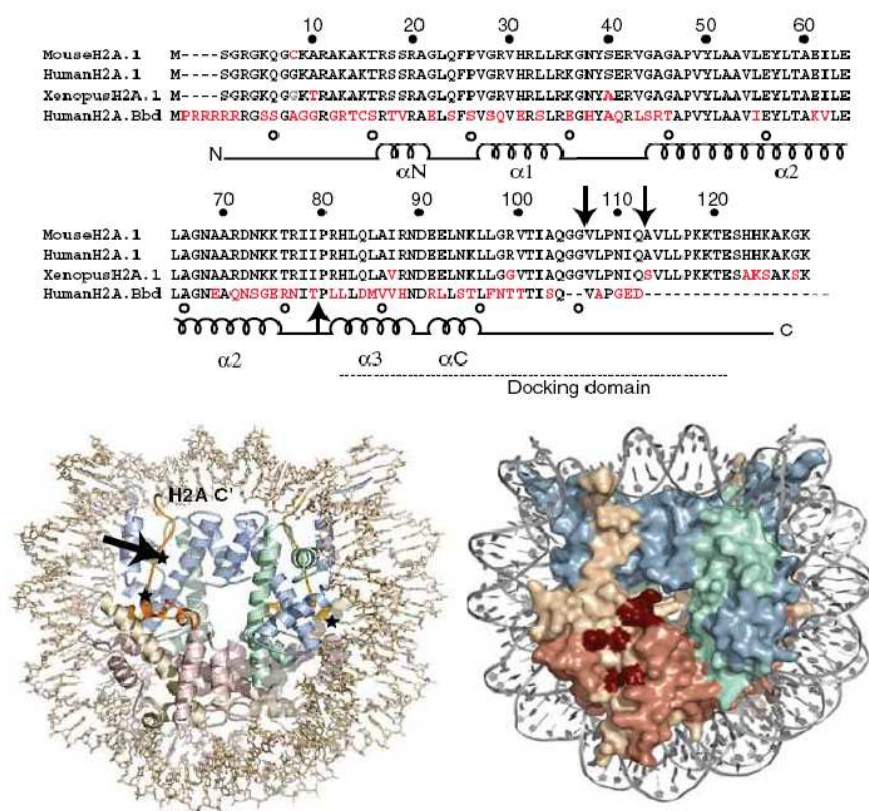
Figure I.11 Sequence and structure of H2A.Bbd Comparison of H2A and H2A.Bbd sequences (on top). Position of anchorage domain of H2A in the nucleosome structure is indicated by black arrow (bottom, left) and surface of nucleosome and position of 'acidic patch' is represented in red (bottom, right). Adapted from Bao et al., 2004 and Caterino and Hayes, 2007.

I.4.2 Posttranslational modifications of histones

Conserved structure of a nucleosome can attain a unique identity by chromatin modifications. The variations in the DNA organization takes place either through histone variants or posttranslational modifications of the amino-terminal tails of core histones. Histone modifications were first described in 1960's (Allfrey et al., 1964). Since then, they have been an important focus of chromatin research since these covalent modifications of histones can regulate gene expression either directly or through recruitment of non-histone effector proteins. Several protein families of histone modifying enzymes and chromatin binding effector proteins have now been recognized. Since the amino-terminal tails of histones protrude out of the nucleosome core, they are accessible to modifying enzymes. These modifications include lysine acetylation, lysine and arginine methylation, serine and threonine phosphorylation, ADP-ribosylation and ubiquitination (Figre I.12, Khorasanizadeh, 2004; Kouzarides, 2007). However, these modifications do not affect integrity of the nucleosome directly, as nucleosome is stabilized by globular regions of the four core histones. "The histone fold" imposes strong accessibility constraint because of which very few modifications are found in the globular domain of core histones eg. methylation of lysin 79 of the histone H3 (H3K79me) (Freitas et al., 2004).

The covalent modifications leads to alteration in electrostatic charge of the histones further leading to change in structural properties of histones and alteration in amino-terminal tail interactions. It is well established that these histone modifications are used as signals by chromatin modifying proteins however, the electrostatic force produced by these modifications might not be sufficient to affect the accessibility of nucleosomal DNA (Polach et al., 2000; Mutskov et al., 1998). Specific proteins are known to bind to the amino-terminal tail of histones and carryout or influence its modification. Two principal protein motifs that play major role in interaction between histone modifications and effector proteins are 'bromodomains' and 'chromodomains', allowing the recognition of acetylated and methylated residues respectively. For example, protein HP1 (heterochromatin protein 1) binds to amino-terminal tail of H3 when methylated at lysine 9 residue via its chromodomain.
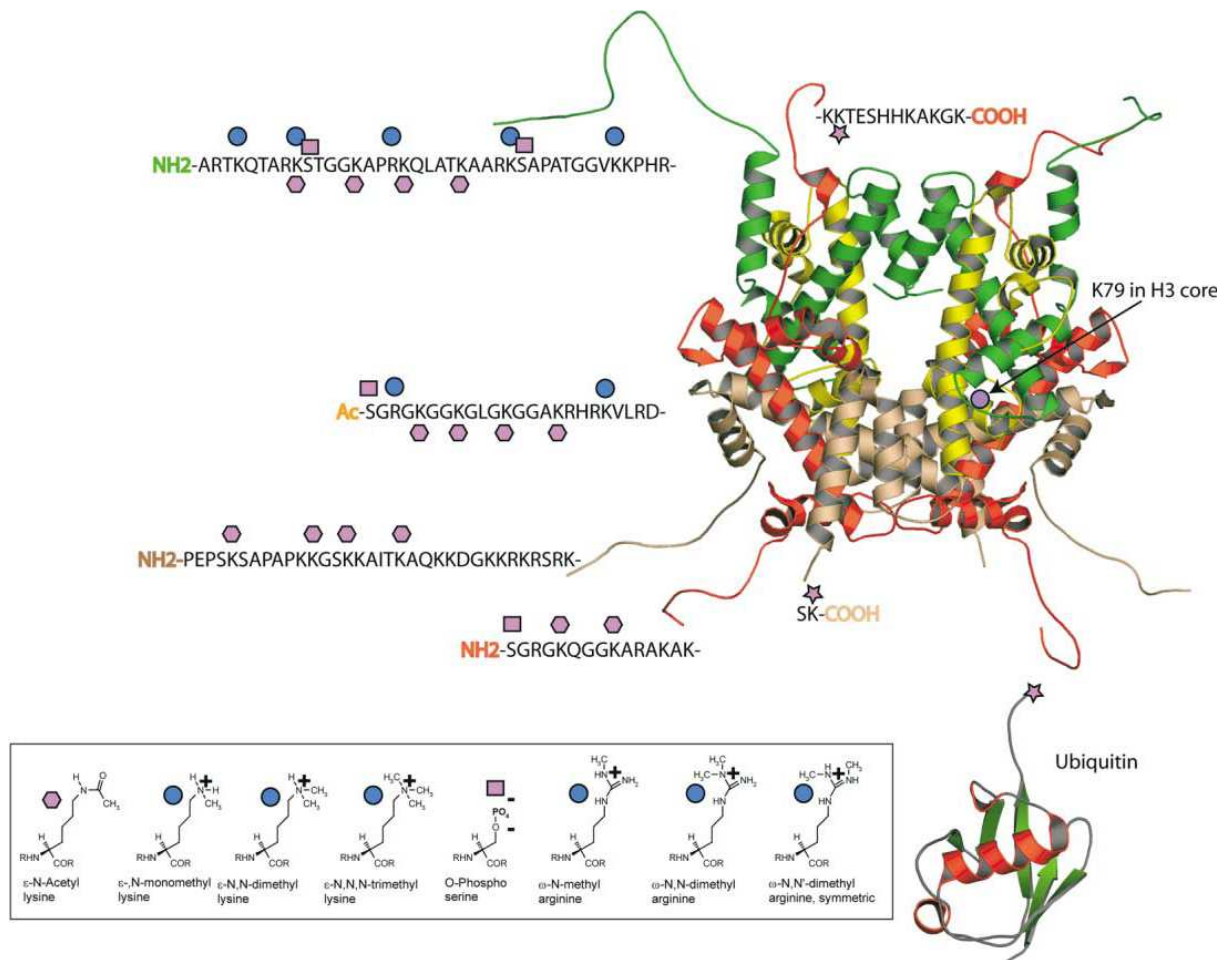
Figure I.12 Posttranslational Modifications of the Core Histones (A) The histone octamer portion of the nucleosome core particle is shown. The sites of modifications are marked. For clarity, the modifications are shown on one copy of each protein. (B) The covalent modifications of the amino acids are shown. Adapted from Khorasanizadeh, 2004.

Different modifications of histone amino-terminal tails constitute the so-called 'histone code'. According to histone code hypothesis a specific combination of histone modifications dictates recruitment of particular transacting factors to accomplish specific functions (Jenuwein and Allis, 2001; Strahl and Allis, 2000; Turner, 2002; Turner et al., 1992). These histone codes can be read individually or as a combination.

I.4.2.1 Histone acetylation

Histone acetylation seems to play an important role in gene expression regulation through chromatin assembly as in general; increased acetylation positively correlates with increased transcriptional activity while decreased acetylation corresponds to transcriptionally repressed state (Fischle et al., 2003; Grunstein 1997; Katan-Khaykovich and Struhl, 2002). Allfrey et al., (1964) first proposed the role of histone acetylation in gene expression regulation however

its clear evidence came with development of antibodies against specific acetylated histones (Turner et al., 1992). Later, Brownell et al., (1996) and others identified enzymes mediating histone acetylation modifications. Now, histone acetylation has been recognized as a dynamic modification of histone controlled by two antagonistic reactions mediated by histone acetyltransferases (HAT) and histone deactylases (HDAC).

HATs form multiprotein complexes that display different histone tail specificities. Bromo-domain is present in many of these proteins through which they recognize acetylated histones (Dhalluin et al., 1999; Jacobson et al., 2000). Moreover, these proteins can physically associate with various transcription factors helping them to target the modified histones thus helps in targeting transcription machinery to specific genes. Likewise, many transcription repressors are known to be associated with HDAC's and that complex plays role in gene silencing (Vaquero et al., 2003). Recently, HDACs are also described to be involved in upregulation of gene expression (Kurdistani and Grunstein, 2003; Robyr et al 2002; Wang et al 2002). Besides gene regulation, histone acetylation plays an important role in many other nuclear processes like DNA repair and apoptosis, VDJ recombination and dosage compensation in *Drosophila* (Iizuka and Smith, 2003).

I.4.2.2 Histone methylation

Methylation of lysine or arginine by histone methyltransferases (HMTs) was supposed to be a stable mark and was discovered more than 30 years ago but its functional significance has been recognized only recently (Rice and Allis, 2001). Moreover several demethylating enzymes have now been recognized such as JHDM1 (Schneider and Shilatifard, 2006). Thus like acetylation, even methylation is a reversible posttranslational modification of histones and is associated with transcriptional regulation of genes and epigenetic silencing via heterochromatin assembly. This posttranslational modification has best been described for H3 and H4 (Fischle et al., 2003; Vaquero et al., 2003).

HMTs catalyze transfer of up to three methyl groups from S-adenosyl-methionine to a single lysine residue and PRMTs (protein arginine methyltransferases) can make mono- or dimethylated arginines, either symmetrically or asymmetrically (Kouzarides, 2002). Both, the site of the residue and number of methyl groups attached to it, determine the functional role of the modification (Lachner and Jenuwein, 2002; Lachner et al., 2003). For example, methylation of lysine 4, 36 and 79 of H3 is associated with transcriptional activation (Beisel

et al., 2002; Ng et al., 2003; Santos-Rosa, et al., 2002) while di- and tri-methylation of lysine 9 or 23 of H3 leads to gene silencing (Bannister et al., 2001; Cao et al., 2002; Czermin et al., 2002; Lachner et al., 2001). Heterochromatic regions are especially enriched in methylated histones. HP1 binds to di- and tri-methylated form of lysine 9 of H3 (Bannister et al., 2001; Lachner et al., 2001) but this binding is inhibited at the beginning of phase S due to phosphorylation of its serine 10 residue by AuroraB kinase (Fischle et al., 2005). Another protein polycomb, involved in silencing of homeotic genes during development, recognizes methylation of lysine 27 of H3. These proteins bind to methylated histones through their chromo-domain (Brehm et al., 2004).

I.4.2.3 Other histone covalent modifications

Besides acetylation and methylation, histones can undergo phosphorylation at serine residues e.g. serine 10 and 28 of H3 (Fischle et al., 2003). Several kinases and phosphatases are involved in regulation of histone phosphorylation such as aurora kinase and Glc7/PP1 phosphatase (Hsu et al., 2000). This modification is associated with mitotic chromosome condensation. Besides core histones, the linker histone H1 has also been shown to undergo phosphorylation, methylation and ADP-ribosylation (Godde and Ura, 2008; Villar-Garea and Imhof, 2008). Like H3, methylation of lysine 26 of H1.4 supports HP1 binding whereas phosphorylation of serine 27 blocks this (Daujat et al., 2005). HP1 binding can be blocked by phosphorylation of H1.5 (or H1b) suggesting a simple redundancy between the five phosphorylation sites of this histone (Hale et al., 2006). Phosphorylation of H2A variant, H2A.X has also been well described in DNA repair (Double strand breaks) (Marzluff and Pandey, 1988; Rogakou et al., 1998; Li et al., 2005b).

Further, histones can get ubiquitinated by addition of a 76 aa peptide to lysine residue e.g. lysine 123 of H2B. This modification is a prerequisite for methylation of lysine 4 and 79 of H3 (Briggs et al., 2002; Sun and Allis, 2002). Hence, there seems to be a crosstalk between these covalent modifications and together they make a signature on the chromatin. In addition, a variety of other histone modifications has been described, such as ADP-ribosylation, biotinylation, glycosylation and sumoylation. Role of ADP-ribosylation has been implied in DNA repair (D'Amours et al., 1999; Lindahl et al., 1997). Further role of histone modifications has been implicated in cell ageing (Vaquero et al., 2003).

I.4.3 ATP dependent Chromatin remodeling

As described before, chromatin is the natural substrate for all the DNA related transactions in the nucleus. Even the most fundamental unit of the chromatin, the nucleosome, presents a great hindrance to factors involved in such processes. Besides histone modifying enzymes and incorporation of histone variants, cells use a set of molecular machines which use the energy of ATP to change chromatin structure to overcome this barrier. These enzymes range from single catalytic unit to multi-subunit complexes which may exceed ~1 MDa in mass.

Yeast SWI/SNF complex is the founding member of chromatin remodeling enzymes. Several components of this complex were originally identified in genetic screens searching for genes affecting expression of HO endonuclease that is required for mating type Switching and SUC2, which encodes an enzyme required for Sucrose utilization. The name **SWI**tch genes was derived from identification of *SWI1*, *SWI2* and *SWI3* genes which act as positive regulator of HO transcription (Stern et al., 1984). On the other hand, genes *SNF2*, *SNF5* and *SNF6* were found to positively regulate the expression of SUC2, hence the name **S**ucrose **N**on **F**ermentors (Neigeborn and Carlson, 1984). Ensuing studies showed that all these 5 gene products function together as a complex involved in positive regulation of transcription (Peterson and Herskowitz, 1992; Peterson et al., 1994). Further investigations resulted in the purification of SWI/SNF complex of 11 subunits (1.15 Mda) (Côté et al., 1994). The importance of this complex in context of chromatin was established by studies on mutations which could alleviate the effects of swi mutations (SWI independent or *SIN*). Two chromatin proteins were identified, encoded by genes namely *SIN1* and *SIN2* (Kruger and Herskowitz, 1991). SIN1 was found to be a nonhistone protein similar to HMG 1/2 and SIN2 was shown to encode histone H3. Moreover, an altered chromatin structure of SUC2 promoter was seen in *snf5* mutant strains (Hirschhorn et al., 1992; Kruger et al. 1995). SNF5 is a core subunit of SWI/SNF complex and essential for its assembly and catalytic functions (Geng et al., 2001). In parallel, *in-vitro* studies demonstrated that SWI/SNF is DNA dependent ATPase (Laurent et al., 1993). Furthermore, the SWI/SNF complex was shown to be able to disrupt nucleosome structure and enhance transcription factor binding to chromatin (Côté et al., 1994). The identification of SWI/SNF paved way for subsequent identification of numerous complexes involved in ATP dependent chromatin remodeling.

I.4.3.1 Different classes of Chromatin remodelers

A common feature of all the chromatin remodelers is the presence of a motor subunit ATPase sharing sequence homology with the DEXX-box helicase superfamily 2 (SF2) (Eisen et al., 1995). The helicase related proteins are characterized by presence of 7 separated motifs labelled sequentially I, Ia, II, III, IV, V and VI. The helicases themselves are classified into three superfamilies viz. SF1, 2 and 3 based on their sequence and spacing of the motifs (Gorbalenya et al., 1989). Superfamiy SF2 includes several families like the DEAD Box or DEAH box helicases and the so called family 'Snf2-like' (Caruthers et al., 2002). However, Snf2- like family proteins differ with DEAD or DEAH box members with respect to helicase related motif III and IV where the spacing is significantly elongated. Also, their helicase-related motifs Ia, III, IV, V and VI have a rather conserved characteristic, and they contain a number of other conserved sequence blocks (Flaus and Owen-Hughes, 2001). It is noteworthy that, SNF2 family proteins do not posses a strand separation activity like other helicases, due to absence of a PIN motif which is required for this function (Dürr et al., 2005; Singleton et al., 2007).
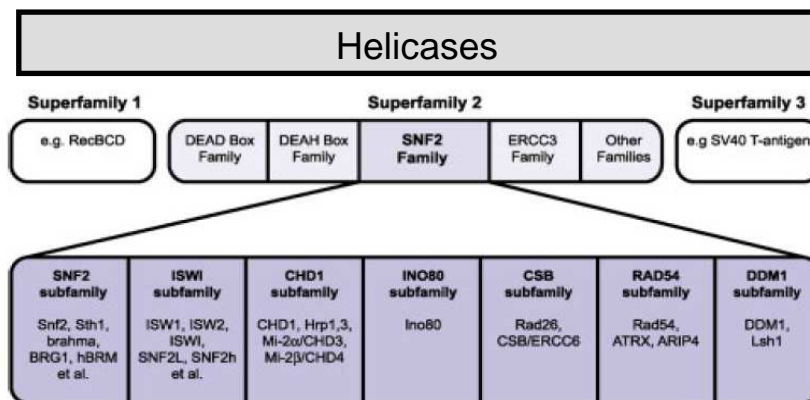
Figure I.13. Classification of SNF2 family ATPases. Adaped from Lusser and Kadonaga, 2003.

The helicase-containing subunits of these enzymes are large multi-domain proteins which contain additional domains like bromodonains, PHD (plectron homology domain), chromodomains, SANT domains and AT hook regions. These additional domains play role in stabilizing interaction of the enzyme with chromatin and also helps in recognizing special histone codes eg. Bromodomain interact with acetylated lysines, AT hook region interact with AT-rich minor groove of DNA and SANT domains interact with histone tails (Aravind and Landsman, 1998; Boyer et al., 2000; Goodwin and Nicolas, 2001). Based on characteristic

domain features and functional properties, chromatin remodelers are subdivided into at least four major subfamilies: SWI2/SNF2, ISWI, INO80 and CHD (Figure I.14).
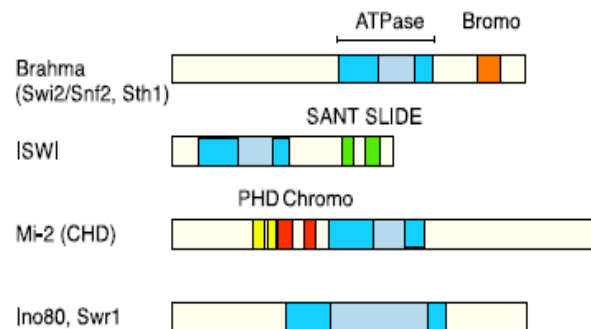
Figure I.14 Classes of ATP dependent Chromatin remodeling enzymes. Adapted from Mohrmann and Varrijzer (2005)

I.4.3.1.1 SWI/SNF family

This sub-group constitutes five members, including the yeast SWI/SNF and RSC complex, the human hbrm and hBRGI complexes, and the *Drosophila* Brahma complex (Cairns et al., 1996; Dingwall et al., 1995; Wang et al., 1996) (See figure I.15 for their subunit composition, homologous and shared subunits).

Yeast		Drosophila		Human	
SWI/SNF	RSC	BAP	PBAP	BAF	PBAF
Swi2/Snf2	Sth1	Brahma	Brahma	BRG1 or hBRM	BRG1
Swi1/Adr6		OSA		BAF250	
	Rsc1, Rsc2, Rsc4		Polybromo		Polybromo/BAF180
	Rsc9*		BAP170*		
Swi3	Rsc8	Moira	Moira	BAF170 & BAF155	BAF170 & BAF155
		BAP111	BAP111	BAF57	BAF57
Swp73	Rsc6	BAP60	BAP60	BAF60a	BAF60a or BAF60b
Swp61/Arp7 Swp59/Arp9	Rsc11/Arp7 Rsc12/Arp9	BAP55	BAP55	BAF53	BAF53
		actin	actin	actin	actin
Snf5	Sfh1	Snr1	Snr1	hSNF5/INI1	hSNF5/INI1
	Rsc5, 7, 10, 13-15				
	Rsc3, Rsc30				
Swp82					
Swp29/Tfg3/TAF30/Anc1					

Figure I.15 Subunit compositions of SWI2/SNF2 family complexes. Adapted from Mohrmann and Verrijzer, 2005.

I.4.3.1.1.1 SWI/SNF

The yeast SWI/SNF complex, considered as the founder for ATP-dependent chromatin remodeling enzymes, is required by many transcriptional activators to enhance transcription in yeast (Peterson and Tamkun, 1995; Winston and Carlson 1992). The yeast SWI/SNF complex consists of 11 subunits viz. SWI1, SWI2/SNF2, SWI3, SNF5, SNF6, SNF11, SWP82, SWP73, SWP29, ARP7 and ARP9 (Cairns et al., 1994; Cairns et al., 1996a; Côté et al., 1994; Peterson et al., 1994; Treich et al., 1995). Mutation in ATP binding domain of Arp7p or Arp9p has shown no phenotypic defect but their deletion mutants are unviable or show reduced growth (Cairns et al., 1998). Thus, the actin-related proteins Arp7 and Arp9 are suggested to share structural but not functional similarities with actin and their role has been implicated in interaction with nuclear matrix.

Several of the yeast SWI/SNF components (Swi2p, Swi3p, Snf5p, Swp73p and the Arp subunits) have homologous counterparts that are constituents of other SWI/SNF-like chromatin remodeling complexes. This indicates a functional conservation among these complexes. However some subunits either show homology in a subset of complexes or are unique to their complex. For instance, yeast Swi1p shows homology to the OSA and Baf250p components of *Drosophila* Brahma and hSWI/SNF (complex A) respectively, whereas Snf6p, Swp82p, Swp29p and Snf11p appear to be unique to the yeast SWI/SNF complex (Figure I.15).

Although little is known about the functional role of individual subunits of the SWI/SNF-like complexes, the size and complexity of these complexes suggest that they perform multiple functions. SWI/SNF complex displays various ATP-dependent biochemical activities. Despite the strong homology with the helicases, no strand separation activity is found during the remodeling (Côté et al., 1994; Quinn et al., 1996; Côté et al., 1998). In an ATP-independent manner SWI/SNF like complexes have the ability to bind naked and nucleosomal DNA with high affinity (Côté et al., 1998; Moreira and Holmberg, 1999; Quinn et al., 1996). SWI/SNF binding properties are similar to high mobility group (HMG)-box containing proteins which recognize structured DNA without sequence specificity in a DNA length dependent manner (Côté et al., 1998; Grosschedl et al., 1994; Pil et al., 1993; Quinn et al., 1996).

I.4.3.1.1.2 RSC

RSC (Remodels Structure of Chromatin) is a complex of about 1MDa isolated from yeast on the basis of similarities between its catalytic subunit protein Sth1 and SWI2/SNF2 (Cairns et al., 1996b). The RSC complex is composed of at least 15 subunits (Cairns et al., 1996b; Sanders et al., 2002). In addition to Sth1, several other sub-units of RSC are similar to sub-units of SWI/SNF complex. RSC subunits, Sfh1, Rsc8 and Rsc6 have respective counterparts in SWI2/SNF2, SNF5, Swi3 and Swp73. The two complexes share actin related proteins namely Arp7 and Arp9 (also named Rsc11/Swp61 and Rsc12/Swp59). In addition, at least 6 sub-units Rsc1, Rsc2, Rsc3, Rsc4, Rsc30 and Rsc58 are specific to this complex. Despite their resemblance, the SWI/SNF and RSC complex are not redundant. Unlike SWI/SNF, the RSC complex is essential for mitosis. On the other hand, estimating yields of purification suggests that RSC is at least ten times more abundant in the cell than SWI/SNF (Cairns et al., 1996b). This indicates that RSC could act on many promoters or be involved in several other processes like repair or replication of DNA.

The RSC complex exists in multiple isoforms. Carins et al., (1996b) purified two distinct forms of RSC, using Rsc6 antibody, having a difference of a 90kDa component and called them as RSC and RSCa. The form RSCa was devoid of Rsc3 and Rsc30 (proteins having zinc cluster domain, which may help in targeting to genomic loci) and represented 10 to 20% of the purified complex (Cairns et al., 1996b; Angus-Hill et al., 2001). Also there are two other RSC isoforms, containing either protein Rsc1 or Rsc2. The isoform containing Rsc2 protein is most abundant. Proteins Rsc1 and Rsc2 are highly similar and are not essential. However, the deletion of *RSC1* or *RSC2* genes confers specific growth defects. The simultaneous deletion of the two genes is lethal (Cairns et al., 1999). Rsc1 and Rsc2 proteins therefore share common functions but are not totally redundant and interchangeable. Each contains two bromodomains, bromo-adjacent homology (BAH) domain and an AT hook (Cairns et al., 1999). BAH domain is found in all eukaryotes and is present in the DNA binding regions of a large number of proteins, which are involved in transcriptional regulation (Callebaut et al., 1999). Besides BAH, the proteins Rsc1 and Rsc2 also contain an AT hook. Chromatin immunoprecipitation experiments were performed on both isoforms complexes but they do not reveal major differences in the location of the protein complex containing Rsc1 or Rsc2 (Ng et al., 2002). The RSC complex was generally found to be recruited to Pol III promoters and it was specifically recruited to Pol II promoters by transcriptional activators and

repressors. Moreover RSC plays essential role in cell cycle progression as Rsc3 mutants exhibit G2/M arrest (Angus-Hill et al., 2001).

I.4.3.1.1.3 SWI/SNF complexes in higher eukaryotes

Homology searches with the yeast Swi2/Snf2 ATPase sequence and biochemical studies have led to the identification of SWI/SNF counterparts in higher eukaryotes. The complexes in *Drosophila* and in mammals contain subunits homologous to Swi2/Snf2 Swi3, Snf5, Swp73 and actin-related proteins (Arp7 or 9) (Phelan et al., 1999).

Drosophila has two SWI/SNF-like complexes BAP (Brahma associated proteins) and PBAP (Polybromo-associated BAP). These contain a common catalytic subunit, Brahma, but differ by additional subunits. BAP contains OSA while PBAP contains Polybromo and BAP170 subunits (Mohrmann and Verrijzer, 2005). Similarly, at least two SWI/SNF-related complexes are found in humans as well namely, BRG1/BAF (hBRM-Associated Factors) or hSwi/Snf-A and hBRM/PBAF (Polybromo-associated BAF) or hSwi/Snf-B. The ATPase subunits of the complexes i.e. BRG1 and hBRM are highly homologous to each other and to yeast Snf2 but they appear to be functionally distinct. hBRM is not essential in mice but BRG1 null mutants die in early development and BRG1 heterozygotes are predisposed to tumor development (Bultman et al., 2000). It has been shown that the human Swi2p, Swi3p, and Snf5p homologues constitute the minimal core of subunits that are required for efficient remodeling activity (Phelan et al., 1999). This suggests that the other conserved components (e.g. Swp73p and Arp proteins) are possibly needed to regulate the minimal core remodeling activities. Recently BAF250, yeast Swi1 related, protein has been identified and is found in hSwi/Snf-A but not in hSwi/Snf-B complex (Nie et al., 2000). Thus there can be a closer relationship between human complex A and yeast SWI/SNF and between complex B and RSC. Besides these two major isoforms, many forms of human SWI/SNF are found as hSwi/Snf can acquire tissue-specific subunits (Wang, 2003) or can associate with other factors such as BRCA1 (Bochar et al., 2000), components of histone deacetylase Sin3 complex (Sif et al., 2001) and histone methylases (Pal et al., 2004).

I.4.3.1.1.4 Structural domains in SWI/SNF subfamily complexes

The subunits of SWI/SNF complexes contain several structural domains with histone or DNA binding activity (Figure I.16). The ***ATPase domain*** consists of seven subdomains that forms two lobes called as DEXD and helicase motifs which form a cleft to which DNA binds (Thoma et al., 2005; Dürr et al., 2005). Swi2/Snf2 protein contains a ***bromodomain*** at its C-terminus. Swi1 contains an ***ARID domain*** (AT-rich interaction domain), which forms a helix-turn-helix structure and preferably binds to AT rich DNA. Swi1 belongs to ARID family but exhibits weaker binding affinity to DNA due to changes in key residues that are normally involved in the interaction (Wilsker et al., 2004). ARID domain is also found in Rsc9 subunit, OSA (in *Drosophila*) and BAF250 and BAP170 (in mammals). It is also called as BRIGHT
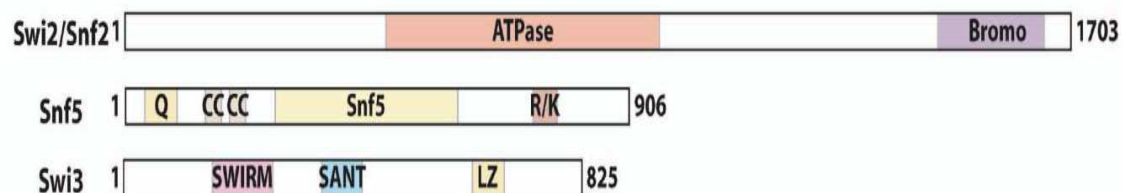
Figure I.16 Domain organizations of SWI/SNF subunits. Bromo means bromodomain, Q is Q rich region, CC stands for coiled coil region, R/K is arginine lysine rich basic region and LZ is lucine zipper motif. Adapted from Gangaraju and Bartholomew, 2007.

domain (B-cell-specific transactivator of IgH transcription) and exhibits both sequence specific as well as sequence independent DNA binding activity (Patsialou et al., 2005; Wilsker et al, 2002; Herrscher et al., 1995; Gregory et al., 1996). Swi3 contains two domains ***SWIRM*** and ***SANT*** (<u>S</u>WI3, <u>A</u>DA2, <u>N</u>-CoR and <u>T</u>FIIIB") which show affinity for nucleosomes and DNA. SWIRM is a conserved domain of about 85 residues and is essential for proper assembly of Swi3 into SWI/SNF complex and activity of SWI/SNF *in vivo*. It is also found in Rsc8, Moira (in *Drosophila*), Ada2 (a component of HAT complex) and LSD1/BHC110 (histone demethylase) (Qian et al., 2005; Aravind and Iyer, 2002; Da et al., 2006). The SANT domain contains about 50 residues and is structurally related to c-Myb DNA binding domain (Mo et al., 2005). It has three alpha helices containing bulky aromatic residues in a helix-turn-helix arrangement and may bind to histones (Grüne et al., 2003). This domain is common among several ATP-dependent chromatin remodeling complexes such as RSC and ISWI and in histone modifying enzymes such as Ada2, Sin3, NCoR (interacts with HDAC) as well as in repressor complexes such as MLL, SMRT and some members of the

polycomb group of proteins (Shi et al., 2005; Boyer et al., 2002; Sterner et al., 2002; Guenther et al., 2001, Yu et al., 2003; Boyer et al., 2004).

I.4.3.1.2 ISWI family

An ATPase, highly similar to brahma, was discovered in *Drosophila* and was named as ISWI (Imitation SWItch) because of its similarity with SWI2 ATPase (Elfring et al., 1994). However, ATPase domains of the two factors can not replace each other hence the name is quite misleading. ISWI type ATPases are characterized by two SANT-like domains in the C-terminal end and absence of a bromodomain (Aasland et al., 1996; Grüne et al, 2003). ISWI complexes preferably bind to nucleosomes containing extranucleosomal DNA than to nucleosome core particles. This might take place via SANT domain (Langst et al., 1999). The ISWI family members appear to take part in a variety of nuclear processes unlike SWI/SNF complexes that are dedicated to transcriptional control. Homozygous null mutation of ISWI is lethal to flies (Deuring et al., 2000). ISWI complexes play role both in transcription activation as well as repression eg. in *Drosophila* it is involved in activation of hsp70 transcription (Okada and Hirose, 1998) and represses specific genes during development. Its developmental role has also been documented in mammals (Stopka and Skoultchi, 2003).

The complexes contain 2 to 4 subunits and are about 200-800 kDa in size. The first members to be discovered of this group were dNURF and dCHRAC in *Drosophila* (Tsukiyama and Wu, 1995; Varga-Weisz et al., 1997). Later other members belonging to this family were identified in different organisms like yeast, mouse and human. (See figure I.17 for their subunit composition, homologous and shared subunits).

S.cerevisae			D.melanogaster			H.sapiens					M.musculus	
ISW1a	ISW1b	ISW2	ACF	CHRAC	NURF	WCRF/hACF	WICH	hCHRAC	RSF	SNF2h/Cohesin	NoRC	mWICH
Isw1*	Isw1*	Isw2*	ISWI*	ISWI*	ISWI*	hSNF2h*	hSNF2h*	hSNF2h*	hSNF2h*	hSNF2h*	mSNF2h*	mSNF2h*
loc3	loc2	Itc1	Acf1	Acf1		hAcf1		hAcf1		Mi2	Tip5/Baz2a	
	loc4						Wstf					mWstf
		Dpb4		Chrac16				hChrac17		Mta1 & 2	p50	
		Dls1		Chrac14				hChrac15		HDAC1 & 2	p80	
					Nurf301				p325	RbAp46		
					Nurf55					RbAp48		
					Nurf38					MBD2 & 3		
										Rad21		
										SA1 & 2		
										Smc1 & 3		

Figure I.17 Subunit compositions of ISWI subfamily members. The catalytic subunit is marked by an asterisk. Subunits which are shared by multiple complexes in the same organism are underlined.

Subunits which are homologous in different organisms by virtue of their sequence are shadowed in grey. Adapted from Gangaraju and Bartholomew, 2007.

Drosophila contains three ISWI complexes namely, NURF (NUcleosome Remodeling Factor), ACF (ATP-utilising Chromatin Factor) and CHRAC (CHRomatin Accessibility Complex). NURF is a four subunit complex where ISWI is found to be associated with BPTF/Nurf301, Nurf55 (pyrophosphatase) and Nurf55 (Tsukiyama and Wu, 1995). Nurf301 forms organizing scaffold of the complex and shares many domains with Acf1 (largest subunit of ACF and CHRAC). Unlike SWI/SNF which gets equally stimulated by nucleosomes and DNA, the ATPase activity of this complex is specifically activated by nucleosomes and not by DNA. NURF interact with N-terminal tail of H4 and this interaction is essential for its ATPase and nucleosome mobilising activity (Georgel et al., 1997). NURF has been shown to activate transcription *in vitro* as well as *in vivo* (Mizuguchi et al., 1997; Badenhorst et al., 2005). The ACF complex contains ISWI and Acf1, a bromodomain and PHD finger protein (Ito et al., 1999). This complex can deposit histone octamers along the DNA in presence of another histone chaperon NAP1 and facilitates regular spacing of nucleosomes in an array (Ito et al., 1997; Fyodorov et al., 2004). However, it also possesses nucleosome sliding activity and can activate transcription (Eberharter et al., 2001). CHRAC is very closely related to ACF. Besides ISWI and Acf1 it also contains two histone fold containing proteins CHRAC-14 and CHRAC-16 (Varga-Weisz et al., 1997). These additional subunits play role in early *Drosophila* development (Corona et al., 2000). Like ACF, it can also make nucleosomal DNA accessible by sliding as well as it generates nucleosome arrays with regular spacing. ISWI complexes are essential for viability and are associated with numerous nuclear processes in *Drosophila* (Corona and Tamkun, 2004).

In yeast, S. *cerevisiae*, two ISWI genes- *ISW1* and *ISW2* have been identified based on their homology with dISWI (Tsukiyama et al., 1999). Unlike *Drosophila* and mice, ISWI is not essential in yeast due to presence of these two redundant copies of ISWI. ISW1p forms two distinct complexes ISW1a and ISW1b (Figure I.17, Vary et al., 2003). ISW1a exhibits strong nucleosome spacing activity while ISW1b does not. Isw2p associates with Itc 1p, a 140kDa protein having partial similarity with Acf1. ISW2 also contains two additional smaller subunits Dpb4 and Dls1 that have histone fold domain and are homologous to dCHRAC 14/16 respectively (hCHRAC 15/17 in humans). ISW2 also exhibits nucleosome spacing activity but is not as tightly regulated as in ISW1a. Moreover, ISW2 does not possess any

nucleosome disruption activity (Tsukiyama et al., 1999; Gelbart et al., 2001). Thus, ISW2 can be considered as CHRAC homolog of yeast.

Likewise, several ISWI-containing complexes such as RSF, hACF, WCRF and hCHRAC (reviewed by Längst and Becker, 2001) have been identified in higher eukaryotes including *Xenopus laevis* (Guschin et al., 2000), mouse (Lazzaro and Picketts, 2001) and human (Strohner et al., 2001; Aalfs et al., 2001). These complexes contain homologous counterparts of *Drosophila* proteins for example hCHRAC contains subunits that are conserved in *Drosophila* ISWI complexes: hACF1, the human homologue of *Drosophila* Acf1, a subunit of ACF, and the human counterparts of two novel histone-fold proteins hCHRAC 15 and 17 that are part of *Drosophila* CHRAC. Similar to yeast, two ISWI genes have been identified in humans namely hSNF2L and hSNF2H (Okabe et al, 1992., Aihara et al., 1998). Both the genes encode for proteins with about 70% homology to dISWI. hSNF2H is a member of at least two complexes: RSF (Remodeling and Spacing Factor) and hACF/WCRF (Williams syndrome transcription related Chromatin Remodeling Factor). RSF consists of hSNF2H and a 325kDa polypeptide and it exhibits promoter-specific remodeling and nucleosome spacing activities (LeRoy et al, 1998). On the other hand, in hACF complex hSNF2H is found to be associated with WCRF180/BAZ1A (Bochar et al., 2000). WCRF180 shares all conserved motifs of Acf1 thus hACF exhibits chromatin remodeling activities similar to *Drosophila* ACF complex. Any complex containing hSNF2L has not yet been identified.

Besides the conserved Swi2/Snf2 ATPase domain, several structural domains have been identified in the catalytic and accessory subunits of ISWI complexes such as SANT, SLIDE (SANT-like ISWI domain), HAND and AID (Acf1 Interaction Domain) domains (Figure I.18, Grüne et al., 2003). The SANT and SLIDE domains are connected by highly conserved spacer helix. SLIDE domain mediates binding of the complex to DNA. However, deletion of either SANT or SLIDE domains do not affect binding of the complex to nucleosomes but deletion of SLIDE largely abolished its ATPase activity. Further, deletion of both domains adversely affected nucleosome binding activity of the complex. Acf1 contains WAC (WSTF, Acf1, Cbp146p), WAKZ (WSTF Acf1, KIAA0314, ZK783.4), DDT (DNA binding homeobox and Different Transcription factors), BAZ, two PHD fingers and a bromodomain (Ito et al., 1999). Isw1p and Isw2p of yeast share the same domain organization as that of dISWI except that AID domain is absent in yeast counterpart. Ioc3 of ISW1a complex has no

detectable domain organization, while Ioc2 and Ioc4 of ISW1b complex have PHD and PWWP domains, respectively (Vary et al., 2003).
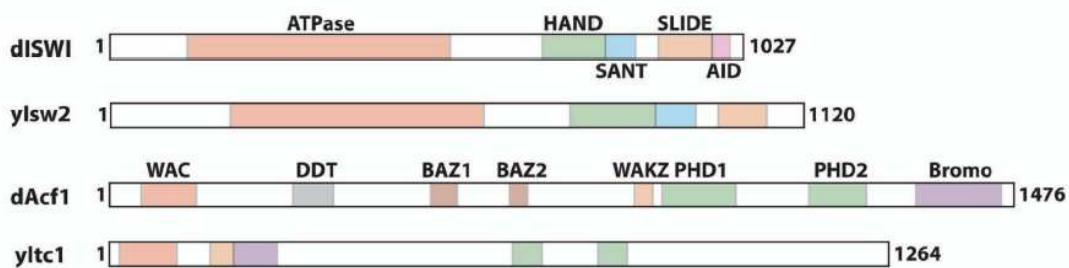
Figure I.18 Domain organization of ISWI subunits. Adapted from Gangaraju and Bartholomew, 2007.

I.4.3.1.3 INO80 family

INO80 is a large complex with 15 subunits and is known to be involved in transcription activation and DNA repair (See figure I.19 for its subunit composition, homologous and shared subunits amongst different species). Ino80p, the largest subunit of the complex contains a conserved but discontinuous ATPase/helicase domain which is split by a large spacer region, contrary to ATPase domain of Swi2/Snf2 and ISWI which are continuous. Also, it contains two conserved regions, TELY motif at the amino terminus and GTIE motif at carboxy terminus (Shen et al., 2000). In addition to Ino80, actin (Act1) and actin-related proteins (Arp 4, 5 and 8) are found to be associated with the complex. Rvb1 and Rvb2 subunits are found to be present in multiple copies per Ino80 molecule and are responsible for 3'-5' helicase activity of the complex. Yeast mutants of INO80 exhibit mis-regulated transcription as well as hypersensitivity to DNA damaging agents implicating its role in both transcription regulation as well as DNA repair. Moreover INO80 complexes have been found to be recruited to double strand break sites through Nhp10 subunit (Morrison et al., 2004; van Attikum et al., 2004).

Another large complex SWR1 (Sw12/Snf2 related) was discovered by three groups at the same time as a large complex that can interact with variant H2A.Z (Krogan et al., 2003; Kobor et al., 2004; Mizuguchi et al., 2004). It contains 14 subunits and shares 4 subunits with INO80 viz. Rvb1, Rvb2, Act1 and Arp4. Moreover like Ino80p, it also contains discontinuous ATPase domain. Like INO80, SWR1 complex has been shown to play

important role in DNA repair and it exhibits a new mode of ATP dependent chromatin remodeling – histone variant exchange. *In vivo*, SWR1 is required for incorporation of H2A.Z variant in yeast genome (Meneghini et al., 2003). Further, Mizuguchi et al., 2004 demonstrated that *in vitro* SWR1 can catalyze replacement of H2A/H2B diamers with H2A.Z/H2B diamers in an ATP-dependent and replication independent manner.

S.cerevisae		H.sapiens
yINO80	ySWR1	hINO80
Ino80*	Swr1*	hIno80*
Arp8		Arp8
Arp5		Arp5
Arp4	Arp4	BAF53a/Arp4
Rvb1	Rvb1	Tip49a
Rvb2	Rvb2	Tip49b
Ies2		hIes2/PAPA-1
Ies6		hIes6/C18orf37
Act1	Act1	Amida
Taf14	Arp6	FLJ90652
Nhp10	Aor1/Swc5	NFRKB
Ies1	Vps71/Swc6	MCRS1
Ies3	Vps72/Swc2	FJL20309
Ies4	Yaf9	
Ies5	Bdf1	
	Swc1/Swc3	
	Swc4/God1	

Figure I.19 Subunit composition of INO80 subfamily members. The catalytic subunit is marked by an asterisk. Subunits which are shared by multiple complexes in the same organism are underlined. Sub units which are homologous in different organisms by virtue of their sequence are shadowed in grey. Adapted from Gangaraju and Bartholomew, 2007.

I.4.3.1.4 CHD family

Like other chromatin remodeling complexes CHD or Mi-2 complexes play important roles in development as mutations in *Drosophila* Mi-2 is embryonically lethal (Khattak et al., 2002). The CHD (Chromodomain Helicase DNA-binding) or Mi-2 complexes contain ATPases with one or more chromodomains. The complexes bind to nucleosomal DNA in a histone tail independent manner through the chromodomains (Bouazoune et al., 2002). In vertebrates, several members of CHD family are found. The first CHD protein (CHD-1) was isolated from mouse as a protein which exhibits features of both Swi2/Snf2 family of ATPases and the Polycomb/HP1 chromodomain family of proteins. But unlike HP1, it is not localised to condensed chromatin (Delmas et al., 1993). CHD1 homolog of *Drosophila* also localizes in transcriptionally active and extended chromatin regions (Stokes et al., 1996). CHD1 of yeast exhibit ATP dependent nucleosome remodeling activity and can reposition nucleosomes however unlike SWI/SNF it can not expose large regions of nucleosomal DNA (Tran et al.,

2000). CHD2 is highly related to CHD1. Yoo et al., (2000) reported another CHD-type ATPase in fission yeast, called as Hrp1, and found it to be involved in chromosome condensation during mitosis. CHD3 (Mi-2a) and CHD4 (Mi-2b) contains two PHD fingers.

Mi-2 complexes are also called as NURD (NUcleosome Remodeling and Deacetylation) due to the subunit composition of the complexes. (See figure I.20 for their subunit composition, homologous and shared subunits among different species). These complexes contain HDAC1/2 as subunits (Kehle et al., 1998; Wade et al., 1998). Besides ATPase and HDAC modules, two additional proteins are found in the human NURD complexes: MTA-1 and MTA-2 (Metastasis Associated Antigens). MTA-2 is a 70 kDa protein and is highly associated to MTA-1 and is essential for efficient deacetylase activity of the complex (Xue et al., 1998; Zhang et al., 1999). Since hypoacetylated histones are known to be associated with repression of transcription, these complexes are thought to be involved in gene silencing. Moreover, it contains another subunit MBD3 which is highly related to methyl cytosine binding protein, MBD2 (Wade et al., 1999). Furthermore, MBD2 itself can associate with the complex and form a chromatin remodeling complex (formally called as MeCP1 complex) which preferentially binds to CpG islands of methylated DNA (Ng and Bird, 1999; Feng and Zhang, 2001). This indicates their role in coordinating histone deacetylation with DNA methylation during gene silencing. In addition, they are also involved in several other repression processes in cells such as repression of homeotic genes during development (Kehle et al., 1998), cell-type specific regulation of genes in lymphocytes (Kim et al., 1999; Cobb et al., 2000) and regulation of cell cycle through human papillomaviruses (Brehm et al., 1999).

S.cerevisae	D.melanogaster		M.musculus		H.sapiens	
CHD1	Mi2	CHD1	CHD1	Mi2	NuRD	ATRX
Chd1*	Chd4* Rpd3	Chd1*	Chd1*	Chd4/Chd3* HDAC1 & 2 RbAp48 Icaros 1,2 & 7 Aiolos	Chd3/Chd4* HDAC1 & 2 RbAp48 RbAp46 MBD3 MTA2	ATRX*
CHD Subfamily is the least characterized and can have uncharacterized proteins						

Figure I.20 Subunit compositions of CHD subfamily members. The catalytic subunit is marked by an asterisk and subunits which are homologous in different organisms by virtue of their sequence are shadowed in grey. Adapted from Gangaraju and Bartholomew, 2007.

I.4.3.2 Targeting of chromatin remodelers

In general, chromatin remodelers do not exhibit any intrinsic DNA sequence specificity hence their recruitment to specific genes must involve other factors which ultimately lead to targeting of the remodeling complexes to specific loci. Several theories and models have been proposed to explain the chromatin remodelers targeting.

Over the past few years three models have been proposed for SWI/SNF targeting (Figure I.21). The 'Catalytic model' proposes that SWI/SNF catalyses transient changes in the chromatin structure randomly and persistent, targeted changes occur only in presence of a transcription factor (Owen-Hughes et al., 1996). This model is insufficient to explain the specificity of SWI/SNF as it is a relatively rare enzyme (Côté et al., 1994) however it can be true for other abundant complexes like NURF and CHRAC, in *Drosophila*. Alternatively, 'Holoenzyme model' was proposed based on its association with RNA polymerase II holoenzyme. However, mutations in holoenzyme do not yield a characteristic Swi⁻ phenotype. Moreover, works of Natarajan et al., (1999) and Yudkovsky et al., (1999) have raised questions against this model as they do not found an obligatory association between Pol II holoenzyme and SWI/SNF. In contrast to the previous two, a relatively simple 'Activator model' was proposed according to which gene specific activators recruit the SWI/SNF complex directly to the target gene. The model was initially supported by Yoshinaga et al., (1992) study wherein they demonstrated SWI/SNF association with glucocorticoid receptor. Further, SWI/SNF has been shown to interact directly with a variety of transcription activators in yeast such as GCN4, SWI5, GAL4-VP16 and GAL4-AH through transcriptional activation domain (Natarajan et al., 1999; Yudkovsky et al., 1999; Neely et al., 1999). Besides yeast, human SWI/SNF also associates with glucocorticoid receptor, *in vivo* (Fryer and Archer, 1998). Moreover, SWI/SNF appears to be recruited by C/EBPβ and collaborates with c-myb to activate myeloid gene transcription (Kowenz-Leutz and Leutz, 1999). Lee et al., (1999) detected hSWI/SNF near β-globin transcription initiation site and the recruitment required erythroid Krüppel-like factor binding site and TATA element. On the other hand, factors binding to cytomegalovirus enhancer were unable to recruit hSWI/SNF.
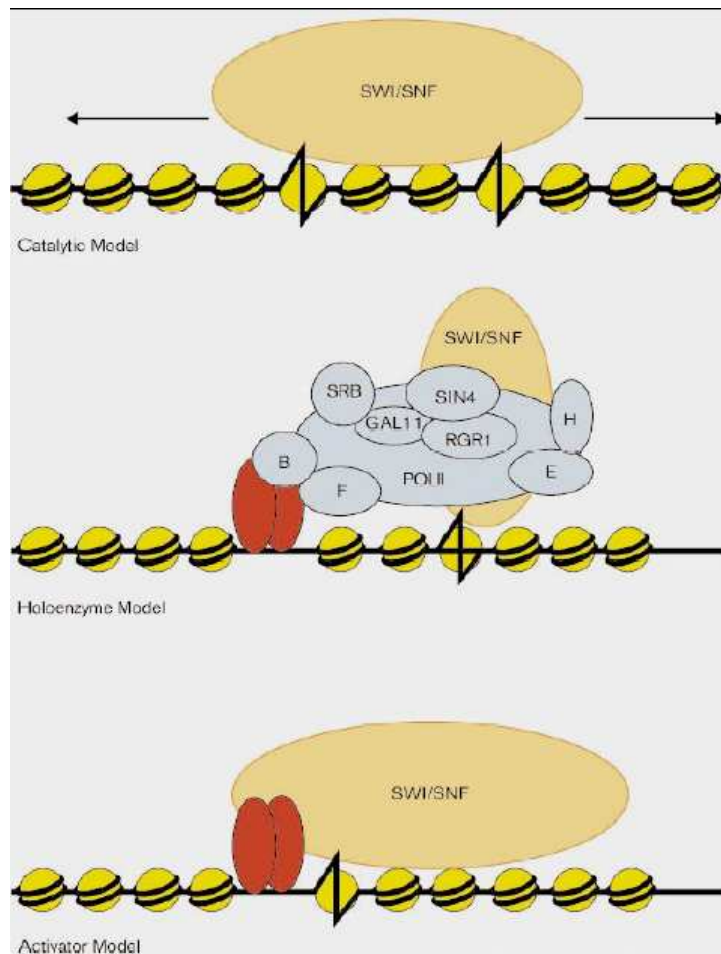
Figure I.21 Models for SWI/SNF recruitment to target genes. Adapted from Peterson and Workman, 2000.

Hence, there is specificity between transcriptional activators and SWI/SNF complex which dictates targeting of the complex to specific loci depending upon the binding site present in the promoter region of the gene. Xu et al., (2006) demonstrated that SWI/SNF protein Brg1 is recruited to the P4.2 promoter by E box–GATA-binding complex and is involved in transcriptional repression in murine erythroid progenitors. Hence the 'Activator model' holds true even in higher eukaryotes.

ISWI-complexes show two modes of binding to chromatin: a basal level of binding globally throughout the genome (catalytic model), and a more target specific interaction (Fazzio et al., 2005). Like SWI/SNF, ISWI also requires presence of sequence specific DNA binding proteins for *in vivo* target specific binding (activator model) (Bachman et al., 2005). Another mode of targeting is seen with the Williams Syndrome Transcription Factor (WSTF) which interacts with PCNA directly to target chromatin remodeling by SNF2H to replication foci (Poot et al., 2004). ISWI complexes can also 'sense' histone modification. They require the

H4 'basic' patch of amino acids R17H18R19 to specifically associate with the target sites on chromatin (Clapier et al., 2002). ISWI is also targeted to nucleosomes containing specific methylation marks, however, the mechanism of interaction with methylated histones is not well understood (Mellor and Morillon, 2004; Santos-Rosa et al., 2003). Another example of specific recognition of histone modifications is the interaction of CHD1 with methylated Lysine 4 of histone H3 (Flanagan et al., 2005; Pray-Grant et al., 2005; Sims et al., 2005).

In summary, chromatin remodeler targeting may occur primarily via interactions with other regulatory proteins or to epitopes on the histones marked by specific modifications.

I.4.3.3 Regulation of chromatin remodeling

Besides targeting of remodeling complexes to required loci, the activities of the complexes themselves must be tightly regulated, because aberrant activity could have deleterious effects on the organization and expression of eukaryotic genomes. This regulation takes place through variety of ways including posttranslational modifications of subunits and changes in subunit composition of the complex or through interaction with secondary messenger molecules and non histone proteins.

I.4.3.3.1 Posttranslational modification of active subunit

Like cell signaling proteins, ATPase subunit of chromatin remodeling complexes are directly regulated by posttranslational modifications especially phosphorylation and acetylation. These modifications may cause a conformational change that can alter mobility of the complex or there can be a monomer-dimer transition of the complex. For example, two subunits BRG1/BRM and SWI3 of hSWI/SNF gets phosphorylated during mitosis so as to inactivate the complex and exclude from chromatin to facilitate chromosome compaction and this is reversed by dephosphorylation as cells exit mitosis (Muchardt et al., 1996; Sif et al., 1998). This reactivated complex then helps to maintain active and open chromatin structure. Similarly, Mi-2 is phosphorylated by Casein Kinase 2 (CK2) in Drosophila cell extracts (Bouazoune and Brehm, 2005). In contrast to BRG1 and BRM, Mi-2 is found to be phosphorylated through out the cell cycle. Dephosphorylation increases its affinity for the nucleosomal substrate, nucleosome stimulated ATPase and ATP-dependent nucleosome mobilization activities. This suggests that constitutive phosphorylation serves to restrain

enzymatic activity and once recruited to chromatin it gets fully activated by an unidentified phosphatase. This presents a possible mechanism to rapidly and reversibly control Mi-2 activity, subsequent to chromatin association. Furthermore, Mi-2 associates with HDAC subunits in the final remodeling complex. The HDAC component of the complex is also a target of CK2 but here phosphorylation upregulates deacetylase activity (Tsai and Seto, 2002).

Like phosphorylation, acetylation of BRM at its carboxy terminus also limits the activity of SWI/SNF complex (Bourachot et al., 2003). This could be because; the modification could alter the structure and thus affect interaction with other molecular partners. The acetylation sites are not found in the highly homologous BRG1 protein. Moreover BRG1 can associate with HDACs (NCoR co-repressor complex) which can help to maintain deacetylated state of BRG1 and thus its catalytic activity (Underhill et al., 2000).

I.4.3.3.2 Subunit composition of the remodeling complex

In general, ATP dependent chromatin remodelers are multi-subunit complexes, consisting of 2 o 15 subunits. The non-ATPase subunits play an important role in regulation of the activity of ATPase subunit. For example, in SWI/SNF complexes, presence of BAF155, BAF170 and SNF5 stimulate the activity of BRG1 and hBRM (Phelan et al., 1999; Geng et al., 2001). Likewise, ACF1 subunit increases the ability of ISWI complexes to assemble regular nucleosomal arrays, it enhances its nucleosome sliding efficiency and alters the direction in which it moves nucleosome on DNA (Ito et al., 1999; Eberharter et al., 2001). Furthermore, interaction of ISWI with ACF1 alters nucleosome structural requirement for the complex to target a locus. ISWI alone requires the histone tail domains of H4, H2A and H3 while in presence of ACF1 only the H4 tail is required (Clapier et al., 2001) and it targets ISWI complex to heterochromatin replication sites (Collins et al., 2002). Also, ACF1-ISWI complex associates with histone-fold proteins (CHARC-15 and CHARC-17 in humans) that facilitate nucleosome sliding and possibly DNA bending (Kukimoto et al., 2004; Hartlepp et al., 2005). Similarly, NURF complex requires NURF301 for efficient nucleosome sliding and targeting (Xiao et al., 2001). Like CHARC, may proteins having ability to bend or stabilize bent DNA are found to be associated with the chromatin remodeling complexes as a subunits and are known to facilitate remodeling activity of the complex e.g. BAP111 subunits of the *Drosphila* BRM complex, BAF57 of SWI/SNF-like complexes in mammals, Nhp10 of

INO80 and Nhp6 of RSC (Papoulas et al., 2001; Chi et al., 2002; Shen et al., 2000; Szerlong et al., 2003).

Moreover, sometimes ATP dependent chromatin remodeling complexes can exist in different forms having distinct subunits composition e.g. RSC exists in two functionally distinct forms (Cairns et al., 1999). Also, BAF can have BRG1 or hBRM as the core motor subunit and accordingly its association with the class of transcription factors is decided and hence targeting of the complex to a promoter depends upon the subunit composition (Bultman et al., 2000; Kadam and Emerson, 2003). In mammals, SWI/SNF complexes are also known to contain tissue-specific subunits and can form additional subcomplexes upon association with other factors like BRCA1 or components of histone deacetylating Sin3 complex. Actin and actin related proteins (ARPs) can dock together different remodeling complexes (Szerlong et al., 2003) and can modulate binding of remodeling complexes to chromatin or nuclear matrix. Moreover, they stimulate ATPase activity and promote complex assembly and stability e.g. Arp4 is essential for INO80, SWR1 and HAT complex, as it recognizes phosphorylated H2A (at ser 129) of damaged DNA and mediates binding of the complexes to the double stranded break region (Downs et al., 2004). Hence, additional subunits regulate ATPase activity of the catalytic subunit along with overall stability of the complex and plays important role in targeting the complex.

I.4.3.3.3 Interaction with secondary messenger molecules

Chromatin remodelers can directly respond to cell signaling pathways by interacting with secondary messenger molecules, most important of them all are lipid inositol 4,5 biphosphate (PIP2) and soluble inositol polyphosphates (IPs). The PHD finger domain of various remodeling complex subunits like BAF and ACF1 have been implicated in interacting with phosphoinositides (Gozani et al., 2003). IPs have been found to regulate the activity of several yeast nucleosome remodeling complexes which have been implicated in regulating genes involved in inositol and phosphate metabolism (Rando et al., 2002; Shen et al., 2003; Steger et al., 2003).

I.4.3.3.4 Interaction with non-histone proteins

Besides histones, HMG (High Mobility Group) proteins are found abundantly in chromatin and are known to play an important role in regulation of gene transcription in response to rapid environmental changes. They are divided into three groups: HMGB, HMGA and HMGN. Like linker histone H1, HMGBs can interact directly with nucleosomes but both have contrary effects. HMGBs loosen up the DNA and make it more accessible to remodeling complexes and transcription factors (Wu and Travers, 2004) whereas, H1 limits spontaneous nucleosome sliding and remodeling by SWI/SNF complex (Ramachandran et al., 2003; Hill and Imbalzano, 2000). Transient interaction of HMGB1 with nucleosomal linker DNA enhances the ability of ACF to bind nucleosomal DNA and accelerates its sliding activity (Bonaldi et al., 2002). Hence, HMGB1 acts as a DNA chaperone and facilitates the rate-limiting DNA distortion during nucleosome remodeling. Moreover, HMG-type proteins also play important role in chromatin remodelers targeting by facilitating interactions between remodeling complexes and site-specific targeting factors e.g. targeting of hSWI/SNF containing BRG-1 to HIV-1 promoter by ATF-3 transcription factor requires HMGA1 (Henderson et al., 2004). Another level of regulation is added to this system by posttranslational modifications of HMG proteins (Bergel et al., 2000; Munshi et al., 2001).

Another group of non-histone proteins that facilitate ATP-dependent nucleosome remodeling are 'histone chaperones' e.g. ASF-1, a histone chaperone, has been reported to cooperate with Brahma remodeling complex in Drosophila (Moshkin et. al., 2002). Another interesting example of regulation of remodeler activity comes from the observation that Nucleolin, a nucleolar protein which also possesses histone chaperone activity, has been shown to greatly enhance SWI/SNF and ACF dependent remodeling (Angelov et. al., 2006).

I.4.3.4 Functions of ATP dependent chromatin remodelers

ATP-dependent chromatin remodeling complexes play an important role in the regulation of all the processes involving DNA such as transcription, replication, recombination and repair (Corona and Tamkun, 2004). Moreover, remodeling factors may also play an important regulatory and architectural role in the maintenance of higher order structure of chromatin (Varga-Weisz and Becker, 2006; MacCallum et al., 2002). Hence they have an impact on the cell fate during cell division and differentiation. The roles of chromatin remodelers have been

well documented in various cellular processes including development, cell cycling and some disease mainly carcinogenesis. A snapshot of functions of ATP-dependent chromatin remodeling complexes is presented in Table I.1.

Table I.1 Biological functions of chromatin remodelers. Chromatin complexes carry out various functions in different organisms. The table summarizes their functions in different species. *Sc*, *Saccharomyces cerevisiae; Dm*, *Drosophila melanogaster*; *Hs*, *Homo sapiens*; *Based on data from many species; *At*, *Arabidopsis thaliana*. Adapted from Saha et al., 2006.

Remodeling Complex	Biological functions	References
SWI/SNF family		
Sc SWI/SNF	Pol II activation	Hirschhorn et al., 1992; Sudarsanam et al., 2000.
	Elongation	Davie and Kane, 2000.
	Double strand break (DSB) repair	Chai et al., 2005.
	Targeting by activators	Neely et al., 2002; Yudkovsky et al., 1999.
Sc RSC	Pol II regulation	Ng et al., 2002; Angus-Hill et al., 2001; Moreira and Holmberg, 1999.
	Pol III regulation	Ng et al., 2002.
	Cell signalling	Angus-Hill et al., 2001; Damelin et al., 2002.
	Spindle-assembly checkpoint	Angus-Hill et al., 2001.
	Chromosome/plasmid segregation	Huang and Laurent 2004; Wong et al., 2002.
	Cohesion	Huang et al., 2004.
	DSB repair	Chai et al., 2005
	Cell-cycle progression	Cao et al., 1997
	Targeting by activators	Neely et al., 2002
	Octamer transfer/ejection	Reinke and Horz, 2003; Boeger et al., 2004
Dm Brahma	Pol II regulation	Armstrong et al., 2002
	Development	Zraly et al., 2004; Marenda et al., 2003
	Elongation	Srinivasan S. et al., 2005
Hs SWI/SNF	Tumor suppressor	Roberts et al., 2000; Wong et al., 2000; Hendricks et al., 2004.
	Differentiation	Gresh L. et al., 2005*;* Vradii et al., 2006; de la Serna et al., 2001a.
	Development	Bultman, S. et al.2000; Wang et al., 2004; Lickert, et al., 2004.
	Elongation	Corey et al., 2003.
	Signaling	Zhao et al., 1998.
	Splicing	Batsche et al., 2006.
ISWI-family		
ISWI*	Elongation	Morillon et al., 2003
	Pol II repression	Goldmark et al., 2000; Vary et al., 2003.
	Replication	Bozhenok et al., 2002; Collins et al., 2002.
	X-chromosome regulation	Deuring et al., 2000.
	Cohesion	Hakimi et al., 2002.
	Embryonic development	Stopka and Skoultchi, 2003.
Dm ACF and CHRAC	Chromatin assembly	Ito et al., 1997; Varga-Weisz et al., 1997; Fyodorov and Kadonaga, 2002.
	Nucleosome spacing	Varga-Weisz et al., 1997.
Dm NURF	Transcriptional activation	Badenhorst et al., 2002.

INO80 family		
Sc INO80	DNA repair Pol II activation	Shen et al., 2000; van Attikum et al., 2004; Morrison et al., 2004. Jonsson et al., 2004.
At INO80	Homologous recombination Gene transcription	Fritsch et al., 2004. Fritsch et al., 2004.
SWR1 family		
Sc SWR1	Htz1 deposition	Mizuguchi et al., 2004; Krogan et al., 2003; Kobor et al., 2004.
Dm SWR1	DNA repair	Kusch et al., 2004.
CHD family		
Hs NURD	Transcriptional repression and silencing	Wade et al., 1999; Jones et al., 1998.
Ce NURD	Development	Unhavaithaya et al., 2002; von Zelewsky et al., 2000.

I.4.3.4.1 Regulation of transcription

SWI/SNF complexes in yeast and mammalian cells are involved in the regulation of transcription and are recruited to promoters by sequence specific transcription factors (Kadam and Emerson, 2003; Prochasson et al., 2003). The chromatin remodeling activity then facilitates binding of both specific and general transcription factors, and it also facilitates the binding of factors involved in repression, such as HDACs. It is important to recognize that chromatin remodeling *per se* does not determine whether transcription will be activated or repressed, although SWI/SNF activity has so far mostly been associated with activation several examples of transcriptional repression have also been documented (Moehle and Jones, 1990; Trouche et al., 1997; Moreira and Holmberg, 1999; Murphy et al., 1999). A well studied aspect of SWI/SNF mediated transcriptional activation is the interaction between SWI/SNF and nuclear hormone receptors. The GR (glucocorticoid receptor) recruits SWI/SNF to the MMTV promoter, resulting in increased DNA accessibility that is essential for transcriptional activation (Fryer and Archer, 1998; Ostlund Farrants et al., 1997). The complex regulates transcription either directly or via various regulatory proteins e.g. proteins Pho2 and Pho4 activatetranscription of *PHO5* gene (Sudarsanam et al., 2000).

The loss of function mutation of SWI/SNF leads to various different phenotypes including poor growth, inability to use particular carbon sources, and a defect in sporulation, however, it

is not required for viability (Cairns, 1998). Further, studies involving DNA chips revealed that only a small fraction (3-6 %) of genes depends on SWI/SNF for their transcription and hence it does not play a general role in transcription of the whole genome (Holstege et al., 1998; Sudarsanam et al., 2000). SWI/SNF regulated genes are distributed throughout the genome and are not concentrated to a particular chromatin region. In general, SWI/SNF appears to be involved in regulating pol II genes. The human SWI/SNF complex can facilitate binding of TBP (TATA binding protein) to a nucleosomal TATA element (Imbalzano et al., 1994). It is highly interesting that remodeling by SWI/SNF is not only promoter specific, but also varies depending on cell type. For example, BRG1 expression in SW13 cells strongly induces *cd44, osteonectin* and *csf1*, while BRG1 expression in ALAB cells induces only *osteonectin* (Hendricks et al., 2004). One explanation for this cell type specific dependence is that additional transcription factors besides SWI/SNF are simply not expressed in the cells, preventing SWI/SNF mediated promoter activation/stimulation. Alternatively, epigenetic patterns established during development could result in the same promoter having tissue specific chromatin topology and, consequently, to require different promoter activities for transcriptional activation or repression. Depending on tissue origin, the same gene could subsequently show variations in SWI/SNF dependency for its expression in different cell types.

Like SWI/SNF, RSC complex is also involved in controlling the transcription. However in contrast with human and yeast SWI/SNF complexes, RSC has not been co-purified with RNA polymerase II of yeast (Cairns et al., 1996, Wilson et al., 1996; Neish et al., 1998). Moreover, it is much more abundant than SWI/SNF and genome-wide location analysis indicates that the yeast nucleosome-remodeling complex RSC has about 700 physiological targets especially tRNA promoters, suggesting that the complex is recruited by the RNA polymerase III transcription machinery. At RNA polymerase II promoters, RSC specifically targets several gene classes, including histones, small nucleolar RNAs, the nitrogen discrimination pathway, nonfermentative carbohydrate metabolism, and mitochondrial function. At the histone *HTA1/HTB1* promoter, RSC recruitment requires the Hir1 and Hir2 corepressors, and it is associated with transcriptional inactivity. Furthermore, RSC binds to promoters involved in carbohydrate metabolism in response to transcriptional activation, but prior to association of the Pol II machinery. Hence, the RSC complex is generally recruited to Pol III promoters and it is specifically recruited to Pol II promoters by transcriptional activators and repressors (Ng et al., 2002).

Whole-genome analysis of gene expression in *rsc3* and *rsc30* mutants indicated that RSC affects the expression of ribosomal protein and cell wall genes (Angus-Hill et al., 2001). However, it is unclear whether these transcriptional effects are directly or indirectly mediated by RSC. Localization of Rsc9 on the genome indicated a relationship between genes targeted by Rsc9 and genes regulated by stress (Damelin et al., 2002). Rsc9 is involved in both repression and activation of mRNAs. Another interesting example of gene repression by RSC is yeast CHA1 (Moreira and Holmberg, 1999). This gene is strongly induced when the cells are grown in the presence of serine/threonine rich media. In the absence of Sth1p/Nps1p (a homolog of Swi2p/Snf2p) or of Swh3p (a homolog of Swi3p), expression of CHA1 in non-induced cells is increased to a level comparable with that of fully induced cells. These transcriptional changes are correlated with disturbances of the chromatin structure of the promoter. Hence, RSC complex represses CHA1 basal transcription by establishing and maintaining a repressive nucleosome structure.

Other examples of transcriptional repression by chromatin remodelers come from NURD complexes (containing the CHD-type ATPase Mi-2), which have both nucleosome remodeling and histone deacetylation activities (reviewed in Bowen et al., 2004). Also, NoRC (for Nucleolar chromatin Remodeling Complex) containing the ISWI-homologue SNF2H is involved in the repression of PolI transcription through the recruitment of the SIN3/HDAC co-repressor to the ribosomal DNA promoter (Santoro and Grummt, 2005).

I.4.3.4.2 Regulation of cell cycle

RSC is the only remodeling complex that is required for cell viability (Cairns et al., 1996). NPS1/STH1 gene (encoding RSC) of *Saccharomyces cerevisiae* is shown to be essential for mitotic growth, especially for the progression through the G2/M phase. The G2/M arrest conferred by four temperature-sensitive (ts) *RSC* mutations suggests a requirement for RSC function in cell cycle progression (Tsuchiya et al,. 1992; Cao et al., 1997; Du et al., 1998; Angus-Hill et al., 2001).The homozygote of the temperature sensitive nps1 mutant, nps1-105, showed reduced and delayed levels of sporulation, accompanied with a notable decrease and delay of the expression of several early meiotic genes (IME2, SPO11 and SPO13) (Yukawa et al., 1999). The basis for this G_2/M arrest is unknown, but it depends on the spindle body checkpoint. Mutants nps1-105 and sth1-3TS are sensitive to drugs destabilizing microtubules

(Tsuchiya and al., 1998; Chai et al., 2002). In the mutant nps1-105, the chromatin structure around the centromere is disrupted. Tsuchiya et al., (1998) digested the centromeric regions by nucleases and restriction enzymes and found a change in the digestion profile. This alteration is apparently not due to a loss of nucleosomes in centromeric regions. In addition, a recent study showed the existence of genetic and physical interactions between RSC and components of the kinetochore (Hsu et al., 2003). It is localized at centromeres and plays a role in the separation of mitotic chromosomes (Hsu et al., 2003, Huang et al., 2004). On the other hand, the human counterpart of RSC, SWI/SNF-B is located at the kinetochores (Xue et al., 2000). These data indicate that the RSC complex is involved in cell cycle progression. This function could be due to direct effect of RSC on segregation of chromosomes and the structure of the centromere and indirectly via the regulation of transcription of genes that control the cell cycle.

Besides RSC, other SWI/SNF complexes also interact with a number of regulatory components in the cell cycle machinery thus affecting cell cycling. For example, BRG1 and BAF155 directly interact with cyclin E (Shanahan et al., 1999). Overexpression of BRG1 or BRM in human SW13 cells, which are deficient in these proteins, causes cell cycle arrest and cell senescence due to interaction between BRG1 and the cell cycle repressor protein pRb (Dunaief et al., 1994; Shanahan et al., 1999).

Moreover, levels of various SWI/SNF complexes are also found to be regulated in a cell cycle dependent manner. For example, in humans BRG1 and BRM proteins are both phosphorylated and excluded from condensed chromosomes during the M-phase, but the outcome of the phosphorylation is different. The level of BRG1 remains constant throughout the cell cycle, while BRM level drops down during M phase due to degradation in response to phosphorylation. BRG1 level increases again in late M/early G1 due to dephosphorylation of the remaining protein and, at the same time, *de novo* synthesis of BRM rapidly brings the protein back up to normal levels (Muchardt et al., 1996; Stukenberg et al., 1997). The SWI/SNF subunit BAF155 is also phosphorylated in a cell cycle dependent pattern similar to BRG1 and BRM, and SWI/SNF complexes isolated from M phase cells are inactive in remodeling assays (Sif et al., 1998). Data from yeast show that genes that must be activated in the boundary between M and G1 in the cell cycle, where chromatin is still very condensed, depend on SWI/SNF for transcriptional activation (Krebs et al., 2000).

I.4.3.4.3 Effect on cell differentiation and development

The expression patterns of BRG1 and BRM during embryo differentiation have spatial and temporal tissue specific distribution in mice, in which BRM is specifically expressed as soon as the blastula starts to differentiate (Dauvillier et al., 2001; LeGouy et al., 1998; Randazzo et al., 1994). Similar patterns are seen in developing chicken embryos, emphasizing the role of SWI/SNF complexes in development (Schofield et al., 1999). Moreover, SWI/SNF activity has been associated with differentiation and development of murine muscle, neural, and endodermal and mesodermal cell types (Machida et al., 2001). Other reports have shown that differentiation of NIH3T3 fibroblasts into muscle cells depends on both BRG1 and BRM in cooperation with the transcription factor MyoD. Expression of dominant negative ATPase-deficient forms of BRG1 and BRM severely inhibits this process and specifically represses remodeling of promoters of MyoD-activated genes *in vivo* (de la Serna et al., 2001a; de la Serna et al., 2001b). A new role of ATP-dependent chromatin remodelers ISWI and DOM in stem cell renewal was demonstrated by Xi and Xie (2005). They do this by regulating responses to peptide factor signaling in the stem cell microenvironment ('niche'). In *Drosophila*, ISWI was found to control Germline Stem Cell self-renewal and DOM was shown to be essential for Somatic Stem Cell self renewal. Likewise the remodelers may play a role in stem cell self-renewal in other organisms, including humans, because of their conserved nature. Recently, Osipovich et al., (2007) demonstrated the importance of SWI/SNF complex in initiation of *Tcrb* gene assembly and T cell development. Here they found that recruitment of SWI/SNF to promoters exposes the gene segments to variable-(diversity)-joining (VDJ) recombinase in thymocytes. Together these studies clearly show that chromatin remodelers play an important role in development and differentiation.

I.4.3.4.4 Regulation of DNA replication and repair

Eukaryotic DNA replication is efficiently regulated by chromatin remodeling complexes at various levels (reviewed in Falbo and Shen, 2006). It may help to open up the chromatin to make it accessible to various effector molecules involved in making the origin of replication and also it can keep the chromatin in an open state after the replication fork passes, thereby creating an opportunity for the epigenetic marks to be copied and transmitted to the next generation (Poot et al., 2005). The mammalian ISWI isoform SNF2H has been shown to be required for efficient DNA replication from a viral origin of replication and through

heterochromatin (Collins et al., 2002; Zhou et al., 2005). Likewise, SNF2H may also have a role in chromatin maturation and the maintenance of epigenetic patterns through replication. SNF2H is found to be associated with WSTF, which directly binds to replication factor PCNA (Poot et al., 2004).

Roles of various ATP-dependent nucleosome remodeling factors in DNA repair and recombination have also been identified (Huang et al., 2005; Shaked et al., 2006). In particular, chromatin-modifying complexes, such as the INO80, SWR1, RSC, and SWI/SNF are implicated in DNA repair. The activity of these chromatin-modifying complexes influences the efficiency of the DNA repair process, which ultimately affects genome integrity and carcinogenesis (Morrison and Shen, 2006). Morrison et al., (2004) illustrated the role of INO80 in DNA damage repair through interaction with phosphorylated histone H2A. Moreover, the Ies4 subunit of the remodeling INO80 complex is phosphorylated by ATM/ATR, a necessary step for certain DNA checkpoints to work properly but it does not regulate DNA repair pathways. Detection of a DNA double strand break (DSB) is necessary to initiate DSB repair. Recently, Liang et al., (2007) illustrated an early role of RSC in sensing the cells' DNA damage response. RSC is required for full levels of H2A phosphorylation by facilitating the recruitment of Tel1/ATM and Mec1/ATR to the break site.

I.4.3.4.5 Role in tumor suppression

Several links have emerged between remodeling complexes and oncogenesis however the mechanisms by which remodelers contribute to tumor suppression are not fully understood (Cairns, 2001). Subunits of the mammalian SWI/SNF complex possess intrinsic tumor suppressor function or are required for the activity of other tumor suppressor genes. Mutations in subunits of the remodeling complexes have been associated with various tumors. Many human cancer cell lines show a down regulation of expression or lack expression altogether of several SWI/SNF components and a number of mutations in genes coding for SWI/SNF components have been identified (Decristofaro et al., 2001; DeCristofaro et al., 1999; Reisman et al., 2003; Reisman et al., 2002; Wong et al., 2000). The SWI/SNF subunit Ini1 is strongly connected to cancer development and is mutated or undetectable in several forms of cancer, in particular in pediatric rhabdoid tumors (Roberts and Orkin, 2004; Biegel et al., 1999; Versteege et al., 1998). Specific mutations in BRG1 have been identified in pancreatic, breast, lung and prostrate cancer cell lines (Wong et al., 2000). Moreover, SWI/SNF can

directly interact with various tumor suppressors and proto-oncogenes such as RB, BRCA1, c-Myc and MLL (Bochar et al., 2000; Cheng et al., 1999; Dunaief et al., 1994). F9 murine embryonal carcinoma cells have an absolute requirement for BRG1 (Sumi-Ichinose et al., 1997) and mouse zygotes with a homozygous deletion of BRG1 cannot grow into viable embryos (Bultman et al., 2000). Heterozygous BRG1$^{+/-}$ mice are viable, but the number of offsprings is significantly lower than that for wild type animals. These mice also display an increased predisposition for exencephaly and tumors.

I.4.3.4.6 Conclusions

As described above, the ATP dependent chromatin remodeling constitutes a very important component in regulation of chromatin dynamics. Owing to their role in the fundamental step of modulating DNA accessibility to factors, unsurprisingly, any defect in their function leads to a multitude of effects including serious consequences on important functions like development, DNA damage repair and carcinogenesis.

I.5 Mechanism of ATP dependent chromatin remodeling

Understanding the process of nucleosome remodeling has been an area of intense studies for last 10 years. Numerous biochemical and single molecule studies have provided insights about how this process occurs. However, some questions still remain about the action and finer details of the process (For review see Becker and Horz, 2002; Saha et al., 2006; Cairns, 2007). In the following sections the advances made in the understanding the mechanism of their mode of action is summarized.

I.5.1 Biochemical properties of remodelers

As described before, a common feature of all the remodelers is the presence of a highly conserved ATPase domain. On the expense of ATP, structural alterations are made in the substrate i.e. the nucleosomes. However, different families of remodelers display some common features as well as dissimilarities in their biochemical activities. In the present section, the properties of these remodelers are summarized.

I.5.1.1 Substrate binding

In order to remodel the nucleosomes the chromatin remodelers must recognize their substrates. It is expected from an enzyme that brings about changes in chromatin to interact with DNA. Initial methods to isolate human SWI/SNF included DNA affinity columns indicating towards a nonspecific binding of these complexes to DNA (Kwon et al., 1994). The nucleosome binding activity was evident from initial gel shift experiments with yeast SWI/SNF complex (Côté et al., 1994). Later, the binding properties of both SWI/SNF and ISWI group of remodelers have been established. SWI/SNF remodelers display an affinity of $\sim10^{-8}M^{-1}$ for DNA substrates in an ATP dependent manner (Quinn et al., 1996; Lorch et al., 1998). No difference in binding affinity between DNA and nucleosomes was observed. However, the binding affinity to nucleosomes increases more than three fold in presence of ATP (Lorch et al., 1998). Similarly, RSC remodeling complex does not show any preference for the presence of linker DNA for binding. For ISWI group of remodelers the binding preferences are slightly different. ISWI can bind DNA but with a lower affinity than SWI/SNF group of remodelers (Whitehouse et al., 2003). Moreover, the presence of linker DNA increases the binding affinity towards the nucleosomes (Brehm et al., 2000). It is known that SWI/SNF exhibits a high affinity for four way junction (4WJ) DNA. This property is similar to as shown by HMG-box domain proteins (Quinn et al., 1996). It is noteworthy that this structure is very similar to the entry exit site nucleosomal DNA. Therefore, it was proposed that SWI/SNF and related complexes may bind the entry exit segment of nucleosomal DNA.

Further details about the nucleosome binding of remodelers have been obtained using structural studies using cross linking (Sengupta et al., 2001) and Electron microscopy methods. It has been shown that ISWI contacts three distinct regions within the nucleosomal DNA (i)~10bp of nucleosomal DNA at super helical location 2 (SHL2); (ii) 10 bp region near the entry exit site of DNA and (iii) linker DNA (Kagalwala et al., 2004). SWI/SNF makes contact with ~60 bp of nucleosomal DNA from entry site of DNA to SHL2 (Dechassa et al., 2008). Similarly, RSC has been shown to interact with DNA near the SHL2, however, the interaction data was based on DNaseI footprinting experiment and needs to be confirmed by definitive cross-linking studies (Saha et al., 2006).

On the other hand, structural analyses of yeast RSC and SWI/SNF have been performed three-dimensional micrographs from individual electron micrographs (Smith et al., 2003; Leschziner et al., 2007; Asturias et al., 2002; Chaban et al., 2008; Dechassa et al., 2008). Using a Orthogonal Tilt Reconstruction method (OTR), Leschziner et al., have shown that RSC possesses a deep central cavity, interestingly, of perfect size to fit one nucleosome. Moreover, the authors have also shown the conformational variability in the RSC complex. Similar reconstructions for SWI/SNF also exhibited a cavity sufficient to accommodate at most one nucleosome at a time (Figure I.22). Though the structures are different, the two structures share the apparent feature of the capacity to interact with a single nucleosome in an environment largely surrounded by enzymatic subunits. Although these studies do not give information about the involvement of individual subunits of the complex, they clearly demonstrate that the substrate recognition occurs via involvement of surfaces comprising multiple subunit proteins.
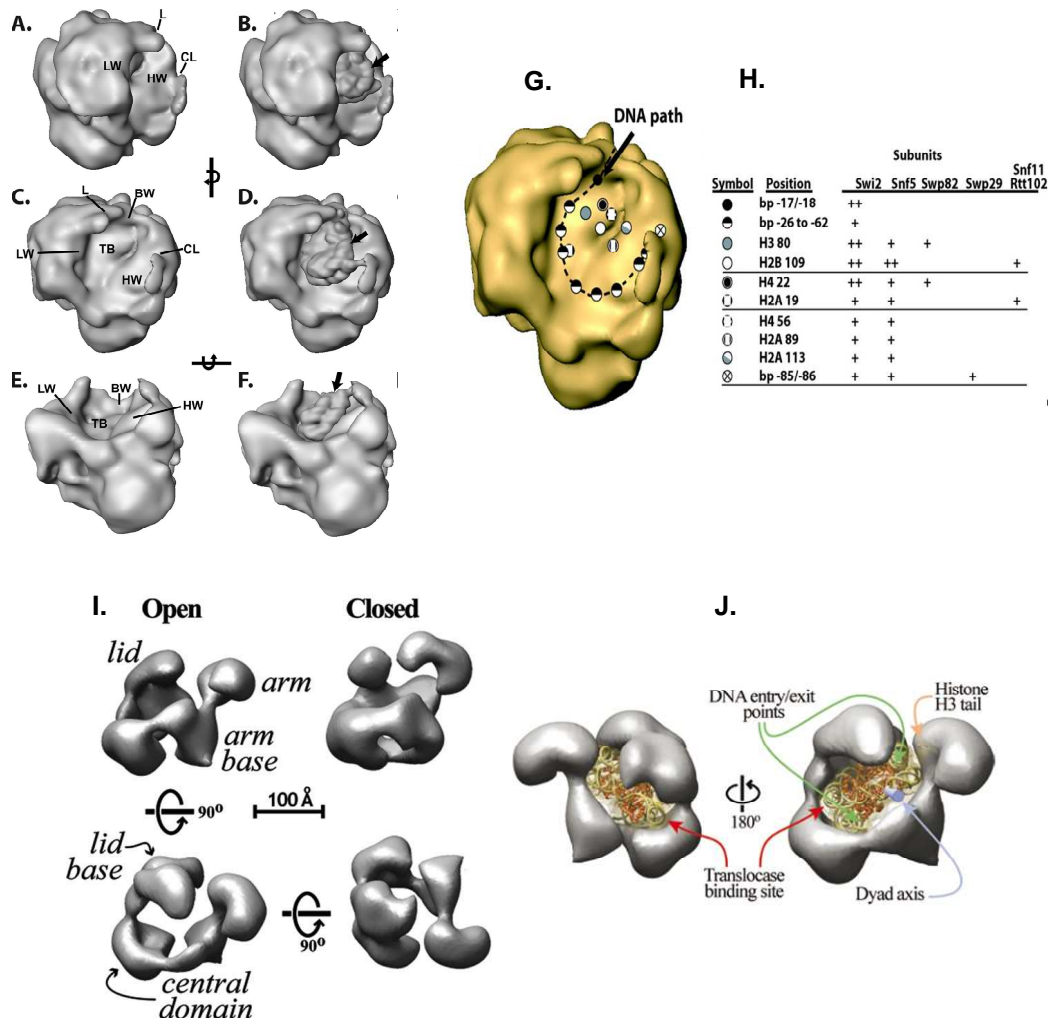
Figure I.22 Structures of SWI/SNF and RSC complexes reconstructed from Cryo-Electron micrographs.

(**A-F**) Cryo-EM reconstruction of SWI/SNF and model of the SWI/SNF-nucleosome complex. Panels A, C, and E show three different views of the SWI/SNF structure obtained from cryoEM. Panels B, D, and F are the models of the SWI/SNF-nucleosome complex obtained by fitting the crystal structure of the nucleosome low pass filtered to 25 Å into the putative nucleosome binding surface of SWI/SNF. Features of the nucleosome binding face of SWI/SNF are a trough whose base (TB) is met by a high wall (HW), a low wall (LW), and a back wall (BW). (G) Model of path of DNA inside the nucleosome binding pocket, (H) SWI/SNF subunits which interact with histones and/or DNA as derived from cross linking studies (Adapted from Dechassa et al., 2008)

(**I**) Reconstructions of two conformers of RSC (**J**) Model of nucleosome binding by RSC. The x-ray crystal structure of the nucleosome was manually fitted into the central cavity of RSC. The nucleosome is shown as a ribbon diagram within a translucent surface representation filtered to 10 Å. The DNA is represented in gold, and the protein is represented in orange. Back (*Left*) and front (*Right*) views of the complex are shown. The entry/exit points of the nucleosomal DNA are indicated with green arrows, the dyad axis (blue cylinder) is indicated with a blue arrow, the histone H3 tail visible in the crystal structure is indicated with an orange arrow, and the binding site for the translocase domain is shown on the DNA with maroon arrows (Adapted from Leschziner et al., 2007).

I.5.1.2 ATP binding and hydrolysis

As the name implies, remodelers require ATP hydrolysis to carry out structural alterations in the nucleosomes. For SWI/SNF remodelers, the ATPase activity is stimulated by single-stranded, double-stranded, or nucleosomal DNA to the same extent (Côté et al., 1994; Cairns et al., 1996). In contrast, ISWI group of remodelers exhibit maximal ATPase activity with nucleosomes while presence of free DNA does not stimulate it (Tsukiyama and Wu, 1995; Georgel et al., 1997). Moreover, ISWI group of remodelers require the N-terminal tail of H4 for full stimulation of their ATPase activity (Clapier et al., 2001; Corona et al., 2002). However, removal of H4 tail does not diminish binding of ISWI, suggesting that this tail may play a role in coupling ATP hydrolysis to conformational changes in the nucleosomes. Under optimal conditions, SWI/SNF remodelers exhibit 2-3 fold higher turnover for ATP as compared to ISWI remodelers. For, both SWI/SNF and ISWI group of remodelers, the stimulation of ATPase activity by DNA shows a length dependence over a limited range of 20-70 bases (Saha et al., 2002; Whitehouse et al., 2003). As mentioned before, although the remodelers belong to SF2 superfamily of helicases they lack double strand displacement activity (Côté et al., 1994). SWI/SNF action does not lead to enhanced sensitivity of nucleosomal DNA to potassium permanganate, indicating a lack of transient duplex unwinding (Côté et al., 1998). However, the helicase regions present in the ATPase subunit are essential for SWI/SNF activity as mutations in these regions diminish the ATPase activity (Côté et al., 1994). Furthermore, the ATPase domains in isolation exhibit limited activity (Corona et al., 1999; Phelan et al., 1999). In summary, different remodelers exhibit both similarity and differences in terms of substrate preference for ATPase activity.

I.5.1.3 Nucleosome disruption activities

ATP dependent remodeling on nucleosomes results in a variety of changes in the nucleosome structure. A common feature of all chromatin remodelers is the ability to enhance accessibility to nucleases or transcription factors. In the following sections, the different outcomes of nucleosome remodeling lead to enhanced accessibility are summarized (See Figure I.23 for a general summary of various outcomes of nucleosome remodeling)

I.5.1.3.1 Generation of superhelical torsion

Since the remodelers belong to SF2 superfamily of helicases it was expected that some helicases like behaviour would be exhibited by these. The evidence was provided by Havas and colleagues (Havas et al., 2000) by testing various chromatin remodelers for the ability to generate super helical torsion in DNA and chromatin substrates. The assay measured extrusion of cruciform from a DNA construct containing an inverted $[AT]_{34}$ repeat. Any superhelical torsion created by the enzyme would result in formation of a cruciform, cleavable by the junction resolving enzyme, T4 Endonuclease VII. It was shown that, SWI/SNF, *Xenopus* Mi-2, ISWI, and recombinant BRG1 were all able to generate superhelical torsion in an ATP dependent manner. However, only BRG1 and SWI/SNF were able to generate torsion on chromatin templates while Mi-2 and ISWI only functioned on nucleosomal template. It must be noted that, however, the generation of superhelical torsion could either be consequence of remodeling or may represent a way by which histone DNA contacts are disrupted.

I.5.1.3.2 Nucleosome sliding

Passive movement of nucleosomes along DNA i.e translational repositioning can occur in response to elevated temperatures or ionic conditions (Meersseman et al., 1992; Pennings et al., 1991). Given the strong interaction between histone octamer and DNA, this process is energetically unfavourable. To achieve this ATP dependent chromatin remodelers use the energy of ATP. In fact, it is a common feature of all the remodelers to mobilize the histone octamer along the DNA (Längst and Becker, 2001). This was first demonstrated in initial studies testing undefined ATP dependent activities in *Drosophila* extracts (Tsukiyama et al., 1994; Varga-Weisz et al., 1995). Later on NURF, CHRAC and ISWI were shown to directionally reposition the mononucleosomes reconstituted on DNA fragments longer than 200 bp in length (Hamiche et al., 1999; Längst et al, 1999). Similarly, yeast and human SWI/SNF complexes as well as the Mi-2 complexes were shown to reposition nucleosomes on short linear as well as small circular plasmid DNAs (Brehm et al., 2000; Gavin et al., 2001; Goschin et al., 2000; Guyon et al., 2001; Jaskelioff et al., 2000; Whitehouse et al., 1999). Additionally, ISWI group of remodelers exhibit the ability to generate regularly spaced arrays (Längst and Becker, 2001). This property was not shared by other families of remodelers indicating that ISWI remodelers may have a role in chromatin assembly.

Some of the ISWI family of remodelers tend to move the nucleosomes to central position on a DNA template, while others seem to randomise nucleosome positioning (Fan et al., 2003; Hamiche et al., 2001). Role of additional subunits have been implicated in such observed behaviour of these remodelers (Yang et al., 2006). On the other hand SWI/SNF group of remodelers shift nucleosomes to the end of the DNA template, away from the thermodynamically preferred position (Flaus and Owen-Hughes, 2003). An interesting feature of SWI/SNF induced nucleosome shifting is that the nucleosomes could be moved ~50 bp beyond the end of the DNA (Kassabov et al., 2003). The ability of SWI/SNF to move the nucleosomes off the ends of DNA could explain some previously reported outcomes of SWI/SNF mediated remodeling. SWI/SNF has been shown to generate di-nucleosome like species or transfer of histone octamer by remodeling mononucleosomes (Lorch et al., 1998, 2001; Schnitzler et al., 1998; Phelan et al., 2000). One can imagine that as the nucleosome is pushed off the DNA fragment, it can be transferred to another DNA or to other slided nucleosome. It must be noted that, however, the abovementioned two outcomes are not the major products of remodeling, at least *in vitro*, and could be generated in the specific reaction conditions used by the authors.

There is some evidence that nucleosome sliding happens *in vivo*. It has been shown that on the interferon beta promoter, which is activated by infection of cells with RNA viruses, the assembly of a complete enhancesome and preinitiation complex occurs lacking only in TBP on the promoter. However, the interaction of SWI/SNF to the promoter is essential for initiation of transcription. Examination of nucleosome positioning before and after transcriptional activation revealed that a nucleosome obscuring TATA sequence was moved to position about 35 bp downstream, thereby permitting TBP to bind and allowing transcription to occur (Agalioti et al., 2000; Lomvardas and Thanos, 2002). Similarly, in yeast Isw2 has been shown to mobilize nucleosomes. The authors used a galactose inducible allele of *ISW2* to study changes in chromatin structure of promoters of test genes. The data suggested that changes were unidirectional and only involved a few nucleosomes (Fazzio and Tsukiyama, 2003).

In summary, ATP dependent chromatin remodelers are able to mobilize nucleosomes *in vitro* as well as *in vivo*. The obvious consequence of nucleosome sliding would be to expose or

shield regulatory regions, thereby permitting or restricting DNA binding factors involved in vital processes like transcription.

I.5.1.3.3 Changes in Nucleosomal DNA conformation: 'Remodeling'

Although all the remodelers have the ability to translationally reposition the nucleosomes, in some cases this activity can not explain the how substantial tracts of DNA are made accessible e.g. in closely spaced nucleosomal arrays. Therefore mechanisms, which could expose DNA sequence within the boundaries of histone octamer without the need for translational repositioning, would facilitate DNA exposure in densely spaced nucleosomal regions. This property is exclusive for SWI/SNF group of remodelers. SWI/SNF family members can increase the DNase and restriction enzyme sensitivity of DNA sites within the nucleosomes (Kingston and Narlikar, 1999; Narlikar et al., 2002). This is achieved even in absence of a linker DNA where nucleosomes could be repositioned. Restriction sites which are close to center of DNA are cleaved with similar rates as those situated at the end of the DNA (Narlikar et al., 2001). Further, site specific cross-linking of DNA to the octamer, which would prevent sliding of nucleosome, does not prevent remodeling by hSWI/SNF (Lee et al., 1999). Moreover, hSWI/SNF and ySWI/SNF can introduce stable topological changes in closed circular arrays (Guyon et al., 2001; Jasekelioff et al., 2000; Kwon et al., 1994). These results can not be explained on the basis of translational repositioning of nucleosomes. Any transient change caused by movement of DNA would be expected to resolve quickly on the unconstrained templates used in the studies.

In summary, remodeling events distinct from nucleosome sliding can be induced by the action of SWI/SNF group of remodelers. Such changes could occur via change in histone octamer conformation or perturbation in the path of DNA around the octamer. It must be noted that most of the aforementioned studies based on nuclease sensitivity assays did not fractionate the remodeled nucleosome and repositioned nucleosome. Moreover, it is known that SWI/SNF is able to translationally reposition nucleosomes even in absence of linker DNA (Kassabov et al., 2003). Therefore, further validation of these events is required and we have, as we shall see in chapter II and III, tried to resolve this issue by fractionation of unmobilized remodeled species.

I.5.1.3.4 Histone H2A-H2B dimer expulsion or exchange

A highly debated question in the field of ATP dependent chromatin remodeling is whether histone octamer is disrupted during this process. Initially, it was suggested that remodeling by SWI/SNF could involve dissociation of H2A-H2B dimer or alteration of the core histone folds (Côté et al., 1994; Peterson and Tamkun, 1995). Histone cross-linking studies have shown that octamer disruption is not a necessary requirement for allowing restriction enzyme access or nucleosome sliding (Boyer et al., 2000). However, some studies suggest that expulsion of dimers can be catalysed by chromatin remodeling enzyme. This was based the fact the remodelers are able to move the nucleosomes beyond the edge of DNA template. It was suggested that this phenomenon would loosen the dimer-tetramer interface and facilitate expulsion or exchange of dimers. Bruno et al., (2003) have shown that SWI/SNF, RSC and ISw1b were able to transfer H2A-H2B dimers from a mononucleosomal substrate to H3-H4 tetramers. Similar phenomenon was observed in an independent study on SWI/SNF (Yang et al., 2007). It was shown that swi3p unit of the SWI/SNF complex was responsible for this action. It must be noted that, however, that these results could occur from the particular DNA template used in the experiment. In both of these studies the DNA template used for nucleosome reconstitution was mouse mammary tumor virus promoter (MMTV) sequence. This sequence is known to be more prone for dimer loss than 5S, another nucleosome positioning sequence (Kelbauskas et al., 2008). There is *in vivo* evidence for this process but only for Ino80 family. An Ino80 family member, SWR1 complex, has been shown to swap H2A.Z-H2B dimers for H2A-H2B dimers (Kobor et al., 2004; Krogan et al; 2003; Mizuguchi et al., 2004).

I.5.1.4 Conclusion

Action of ATP dependent remodelers on nucleosomes results in a multitude of outcomes as enumerated above. These observations have led to proposal of different models of remodeling which could reconcile these outcomes. These models are discussed later in following sections.

Sliding

Remodeled dinucleosome species

Altered DNase I cleavage pattern

Octamer transfer

histones + NAP1

remove protein from array

+remodeler + Topo I

remove protein from array

Assembly and spacing of nucleosomes

Generation of topological changes

$(H2A/H2B)_2$
$(H3/H4)_2$

Dimer loss

Dimer replacement

SNF2 subfamily specific activities

ATPase stimulated by DNA and Nucleosomes

DNase I pattern of chromatin changed

Histone octamer transfer in trans

Dinucleosome formation

Mediates translocation of nucleosomes by ~80 bp

Common activities

Repositioning of nucleosomes in cis

Generation of superhelical torsion

Triple helix displacement

ISWI subfamily specific activities

ATPase stimulated by nucleosomes

ATPase stimulation H4 tail dependent

Chromatin assembly

Mediates modest ~10 bp repositioning of nucleosomes

Figure I. 23 Summary of various biochemical activities of ATP dependent remodelers
Adapted from Narlikar et al., 2002; Lusser and Kadonaga., 2003.

I.5.2 ATP dependent remodelers as DNA translocases

Over the past years, a number of studies on chromatin remodelers have established that their ATPase subunits are ATP dependent DNA translocases (Saha et al., 2002; Jaskelioff et al., 2003; Whitehouse et al., 2003). The evidence for DNA translocation activity was derived from the observations that the remodeler ATPase activity is proportional to the length of the DNA. DNA mini circles induce maximal ATPase activity as they represent a DNA of infinite length. Moreover, the chromatin remodeling enzymes are able to displace the third helix from a short triple helix DNA in ATP dependent manner. So far, SWI/SNF, ISWI and RSC; all of them have been shown to possess a directional 3'-5' translocase activity. It was suggested that SWI/SNF, RSC and ISWI translocate DNA from an internal nucleosomal site located ~2 turns from the dyad. Nucleotide gaps created within this region interfered with nucleosome mobilization (Zofall et al., 2006; Saha et al., 2005).

Further insights about DNA translocase activity of remodelers have come from a series of single molecule experiments involving optical or magnetic tweezers. By combining atomic force microscopy with a magnetic trap, Lia et al., (2006), for the first time, have demonstrated that RSC is able to generate loops on naked DNA. RSC translocated DNA at high speeds (200 bp per second) and for considerable distances (averaging ~420 bp) under conditions of very low tension (0.3 pN). However, the processivity of RSC on free DNA in stopped-flow conditions (bulk measurements) was ~20 bp (Fischer et al., 2007), and bulk length dependent ATPase assays estimated the average translocation distance at ~20–25 bp indicating the occurrence of particularly processive translocation events in the experimental conditions of abovementioned study. Another study by Zhang et al., (2006), using optical tweezer approach, has monitored RSC and SWI/SNF dependent remodeling in real time. Both RSC and SWI/SNF were shown to cause DNA shortening events which were interpreted as formation of loops on the nucleosome surface. DNA was translocated at ~13 bp per second and for distances averaging ~105 bp under a moderately high tension range (3–7 pN). It must be noted that, although these studies have provided direct observation of DNA translocase activity as well as measurement of force applied by the remodelers, a common shortcoming is a bias towards bigger translocation events due to instrument noise. Moreover, the possibility of many remodeler molecules working simultaneously or destabilization of the nucleosomes in typical single molecule experimental conditions can not be ruled out (Claudet et al., 2005).

Further studies are definitely required for elucidation of physical parameters of DNA translocation as well as how the DNA translocation is applied on the nucleosomes.

I.5.3 Models for Nucleosome remodeling

To reconcile the aforementioned outcomes of ATP dependent nucleosome remodeling, two major models have been proposed. Both of these models assume that at a time only a subset of histone DNA interactions are disrupted at any given time and that the energy cost involved in disrupting the histone DNA interaction are compensated, in part, by formation of new bonds. This hypothesis is supported by the observation that SWI/SNF or RSC motors stall at forces above 12 pN while the force required to completely disrupt all DNA histone interactions in the nucleosomes is ~20pN (Zhang et al., 2006). The first model was "Twist Diffusion" model discussed by van Holde and Yager (1985) and readdressed later (van Holde and Yager, 2003). According to this model (See Figure I.24), the migration of DNA around the histone octamer results due to propagation of small twist defects that cause underwinding of the DNA helix which are then diffused around the nucleosome. If the defect collapses back upon itself no net movement of nucleosome occurs. However, if the defect is propagated forward, this results into small slipping steps to occur, resulting in net movement of histone octamer with respect to DNA (van Holde and Yager, 2003). This view is supported by the observations that chromatin remodeling enzymes generate superhelical torsion (Gavin et al., 2001: Havas et al., 2000).

However, there are observations which challenge the universality of this model in all cases. ISWI and SWI/SNF group of remodelers are able to mobilize nucleosomes even in presence of DNA containing nicks, hairpins or gaps (Aoyagi and Hayes, 2002; Längst and Becker, 2001; Saha et al., 2002) which would be expected to interfere with the propagation of twist defect. Nicks in the DNA might dissipate the torsional stress while hairpins might interfere with the rotation of the DNA relative to the nucleosome.

An alternate model, "The bulge propagation or "Loop recapture" model was proposed (Längst and Becker, 2004). In this model it is suggested that that a wave of DNA is released from the histone octamer and propagated along the surface of the nucleosome. The formation of this bulge is the rate limiting step of the remodeling reaction (Strohner et al., 2005). Initial support for this model came from experiments conducted by Aoyagi et al., (2002). H2B was

crosslinked to the DNA, and the remodeling was assessed by sensitivity to nucleases. Interestingly, hSWI/SNF could still increase the sensitivity towards DNaseI even in the absence of nucleosome movement. Using a photo affinity labelling and crosslinking approach Kassabov et al., (2003) have shown that SWI/SNF moves the nucleosomes in increments of ~50 bp while for ISWI the step size was ~10 bp. These were interpreted as the size of the loop or the bulge created by these remodelers. Using this information about the step size authors have tried to explain the observed differences in nucleosome disruption properties of these two remodelers. It must be noted that, however, that new histone DNA crosslinks generated due to remodeling could represent final products of the remodeling rather than reaction intermediates. Another support towards formation of bulge by remodelers come from the fact that remodelers are ATP dependent DNA translocases and are able to pump DNA inside the nucleosome (Saha et al., 2005; Zofall et al., 2006).
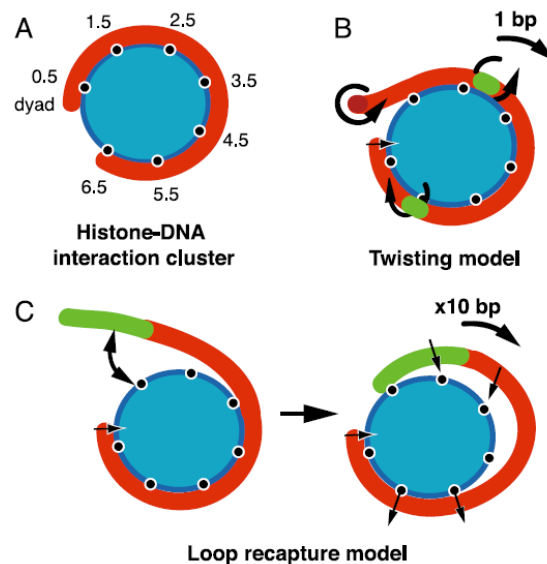
Figure I.24 Proposed models of nucleosome sliding by ATP dependent remodelers.
(A) Schematic drawing of the SHL locations that form DNA– histone interaction clusters. (B) and (C) The essential features of the nucleosome remodeling models. Adapted from Langst and Becker, 2004.

Another model for RSC mediated nucleosome movement was proposed by Saha et at., (2005). Under this model DNA is moved in form of a 1 bp wave from the internal translocation site to the end of the nucleosome. However, if it was the case nucleotide gaps anywhere within this region would interfere with nucleosome mobilization. It was seen that nucleotide gaps created only within or near the translocation site interfere with nucleosome mobilization questioning the validity of this model. Importantly, till date, no direct demonstration of a bulge formation on the nucleosome surface has been done and evidences provided are only indicative.

Moreover, the discrepancies observed in the step size (1-50 bp under different studies) probably resulted from different indirect measurements.

In summary, there is no definite consensus about how the chromatin remodelers work despite of a lot effort put in this direction. In fact, it is only the beginning of our understanding towards the mechanism of ATP dependent chromatin remodelers. Further studies are required to address the questions that these intriguing molecular machines have posed before us.

I.6 Objectives

As we can see, although a lot of effort has been put, a lot of grey areas exist in our understanding of the mechanism of chromatin remodeling. Many questions about the structural features of remodeled nucleosome particles, remodeling intermediates and discrimination between nucleosome remodeling and sliding still remain. Moreover, all the proposed models assume the nucleosome mobilization process to be a non-interrupted, continuing process. Although the bulge propagation model is currently favoured model in the literature, no direct evidence of the existence of a bulge has been provided.

The present study aims to address these issues using yeast RSC and SWI/SNF, one of the best characterized remodelers, as a model system. In chapter II and III we have used a combination of high resolution microscopy and biochemical methods to elucidate the nucleosome remodeling mechanism of RSC and SWI/SNF respectively. Atomic force microscopy approach is employed to obtain precise information about the organisation of DNA on RSC and SWI/SNF remodeled nucleosomes. Cryo-Electron microscopy is used to capture the remodeling products in their native form, as well as to study the conformation of DNA on nucleosomes. The biochemical method like "one pot restriction enzyme assay" allows to measure the accessibility of remodeled nucleosomes with 10 bp resolution. Moreover, special stress is given to discriminate between unmobilized remodeled particles and mobilized nucleosomes. By using these approaches we aim to circumvent the problem in analysis that could arise if an undefined mixture of remodeled and slided nucleosome particles are analysed through classical biochemical methods like restriction enzyme accessibility assay.

Incorporation of histone variants like H2A.Bbd confers the nucleosomes special structural and biological properties. As summarized before, incorporation of H2A.Bbd in nucleosomes results in an open structure of the nucleosomes leading to facilitated factor access to nucleosomal DNA. On the other hand, despite of their open structure, H2A.Bbd containing

nucleosomes are resistant to remodeling by ATP dependent chromatin remodelers like SWI/SNF and ACF. Since most of the structural features of H2A.Bbd nucleosomes have been attributed to its defective docking domain we hypothesised that this apparent inhibition of remodeling could be due to this feature. Using a series of H2A mutant proteins, coupled with biochemical and AFM methods, we have aimed to resolve this issue in chapter IV.

Chapter II: *Manuscript communicated*

Title: A Two-Step Mechanism for Nucleosome Remodeling by RSC: Initial Formation of a Remosome Containing ~180-190 bp DNA Followed by Sliding

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Running Title: A Two-Step Mechanism for Nucleosome Remodeling by RSC

II.1 Summary

We have studied the mechanism of RSC nucleosome mobilization by using high resolution microscopy and biochemical techniques. AFM analysis shows that two types of products are generated during the RSC remodeling: (i) stable non-mobilized particles, termed remosomes, which contain 180-190 bp of DNA associated with the histone octamer and, (ii) mobilized particles located at the end of DNA. Electron-cryo microscopy reveals that individual remosomes exhibit a distinct, variable highly irregular DNA trajectory. The use of the novel "in gel one pot assay" for studying the accessibility of nucleosomal DNA towards restriction enzymes all along its length and DNase I footprinting demonstrate that the histone-DNA interactions within the remosomes are strongly perturbed, particularly in the vicinity of the nucleosome dyad. The data suggest a two step mechanism of RSC nucleosome remodeling consisting of initial formation of a remosome followed by mobilization. In agreement with this model, we experimentally show that the remosomes are intermediate products generated during the first step of the remodeling reaction, which are further efficiently mobilized by RSC.

II.2 Introduction

In all eukaryotes DNA is packaged into chromatin (van Holde et al., 1980), which exhibits a repeating structure with a fundamental unit, the nucleosome, consisting of an octamer of core histones (two each of H2A, H2B, H3 and H4) around which 147 bp of DNA is wrapped. Nucleosomes constitute a barrier for several processes including transcription, repair and replication (reviewed in (Beato and Eisfeld, 1997)). Cells use three main strategies to overcome this barrier: post-translational histone modifications (Strahl and Allis, 2000), chromatin remodeling complexes (Becker and Hörz, 2002) and histone variants (Boulard et al., 2007).

Remodeling complexes are large protein assemblies, consisting of an ATP-requiring DNA translocase of the SWI/SNF family associated with variable numbers of subunits (Becker and Hörz, 2002). According to the type of ATPase, the remodeling factors are classified in at least four distinct groups: the SWI2/SNF2, ISWI, CHD and INO80 families (Bao and Shen, 2007). These four main groups of remodelers also exhibit distinct biochemical properties and specific remodeling characterisitics. A general property of the remodelers is their ability to mobilize the nucleosome without disruption or trans-displacement of the histone octamer (Längst et al., 1999). In addition, the remodelers belonging to the SWI/SNF group can efficiently alter histone-DNA interactions and even evict the histone octamer from DNA (Lorch et al., 1999). It has been also shown that the recently identified Swr1 remodeling complex, which belongs to the INO80 group, possesses novel properties and is implicated in the exchange of the histone variant H2A.Z (Mizuguchi et al., 2003). Interestingly, the presence of the histone variants mH2A and H2A.Bbd interferes with the ability of chromatin remodelers to mobilize these variant nucleosomes (Angelov et al., 2004; Doyen et al., 2006a; Doyen et al., 2006b).

The yeast RSC (Remodels Structure of Chromatin) complex belongs to the SWI2/SNF2 family (Cairns et al., 1996). It is abundant, essential for viability and comprises 15 subunits. RSC is involved in several processes including transcriptional activation, DNA repair and chromosome segregation (Cairns et al., 1999, Huang and Laurent, 2004; Chai et al., 2005). The structural analysis of RSC reveals the presence of a central cavity within the complex sufficient for binding a single nucleosome (Leschziner et al., 2007; Asturias et al., 2002). This

model was recently confirmed by the cryo-EM determined structure of a RSC-nucleosome complex (Chaban et al., 2008). The binding of the nucleosome in the RSC cavity could allow a partial separation of the DNA from histones while maintaining their mutual proximity (Asturias et al., 2002).

It should be noted that despite many efforts, neither the mechanism of the remodeling assembly action nor the conformation of the remodeled nucleosomes are yet established (reviewed in (Eberharter and Becker, 2004; Gangaraju and Bartholomew, 2007). It is, however, clear that the chromatin remodelers exhibit a DNA translocase activity (Lia et al., 2006; Zhang et al., 2006). The reported biochemical data have led to at least two models for chromatin remodeling (Gangaraju and Bartholomew, 2007). According to the first model, initially proposed for the remodeler RSC, DNA moves in 1 bp waves on the histone octamer surface (Saha et al., 2005). According to the second model, proposed for both SWI/SNF and ISW2 remodelers, a DNA loop is formed on the nucleosome surface, which further allows the sliding of the histone octamer (Längst and Becker, 2001; Zofall et al., 2006). Recently it was inferred from data from experiments with optical tweezers that, in contrast to the biochemical reports, RSC is able to generate a loop with average size of about 110 bp at the dyad axis of the nucleosome. This loop was proposed to be a prerequisite for the mobilization of the nucleosome (Zhang et al., 2006). Note that each of these models implicitly assumes that the nucleosome-induced mobilization is a non-interrupted, continuing process, not requiring the dissociation of the remodeler from the nucleosome. Importantly, no direct experimental evidence for the existence of a remodeler-induced DNA loop on the nucleosome surface has been reported.

In this work we show that RSC uses an intriguing two-step mechanism for nucleosome mobilization. The first step consists of pumping of 15-20 bp of the DNA of both linkers towards the centre and the generation of stable non-mobilized remodeling intermediate containing ~ 180-190 bp DNA associated loosely with the histone octamer. During the second step, the mobilization of the histone octamer is achieved. The physiological relevance of such a RSC nucleosome remodeling mechanism is discussed.

II.3 Results

II.3.1 RSC generates stable non-mobilized nucleosome-like particles associated with 180-190 bp DNA

To study the mechanism of nucleosome mobilization by RSC we used reconstituted nucleosomes. Briefly, recombinant core histones were purified to homogeneity and nucleosomes were reconstituted on a 255 bp 601 positioning sequence (Supplementary Figure II.S1). The reconstitution, under the conditions used, was very efficient since no free DNA was detected in the nucleosome reconstituted samples (Supplementary Figure II.S1). Note that reconstitution on the 255 bp 601 fragment generates a precisely centrally positioned nucleosome with 52 bp and 56 bp free DNA arms, respectively (results not shown). The gel-shift assay shows that RSC was able to efficiently mobilize the reconstituted particles in the presence of ATP, demonstrating that the reconstituted particles are *bona fide* substrates for this remodeler (Supplementary Figure II.S1).

Once the reconstituted particles were characterized, we next used AFM to study the organization of the nucleosomes upon incubation with RSC. AFM permits the simultaneous determination of the nucleosome position on the DNA and the length of DNA wrapped around the histone octamer (Montel et al., 2007). This makes this technique extremely useful for characterizing the chromatin remodeler-induced nucleosome mobilization through the evolution of nucleosome position and wrapped DNA length mapping (Montel et al., 2007). In our experiments the APTES-mica surface was functionalized so as to trap the 3D conformation of the nucleosomes (Valle et al., 2005) and the parameters of interest were obtained by using a specially designed algorithm, which allows the analysis of several hundred nucleosomes in each AFM experiment and makes the results statistically significant (see Materials and Methods section and Montel et al., 2007).

Figure II.1 shows a series of representative images for the nucleosomes incubated for 30 minutes in the absence of RSC (control sample, first row) or in the presence of RSC (2nd, 3rd and 4th rows). In the control sample, the nucleosome core particle (pink part of the structure) is clearly distinguishable from the free DNA "arms" (labeled in yellow) and the histone octamer is centrally positioned. Upon incubation with RSC (in the presence of ATP) three

different groups of structures were observed. The organization of the first group (2nd row) is indistinguishable from the control sample (Figure II.1, compare the images of the 1st row with that of the 2nd row). The second group (3rd row) exhibited shorter DNA arms than the control and the third group consisted of completely slided nucleosomes with the histone octamer located at one end of the DNA (4th row).
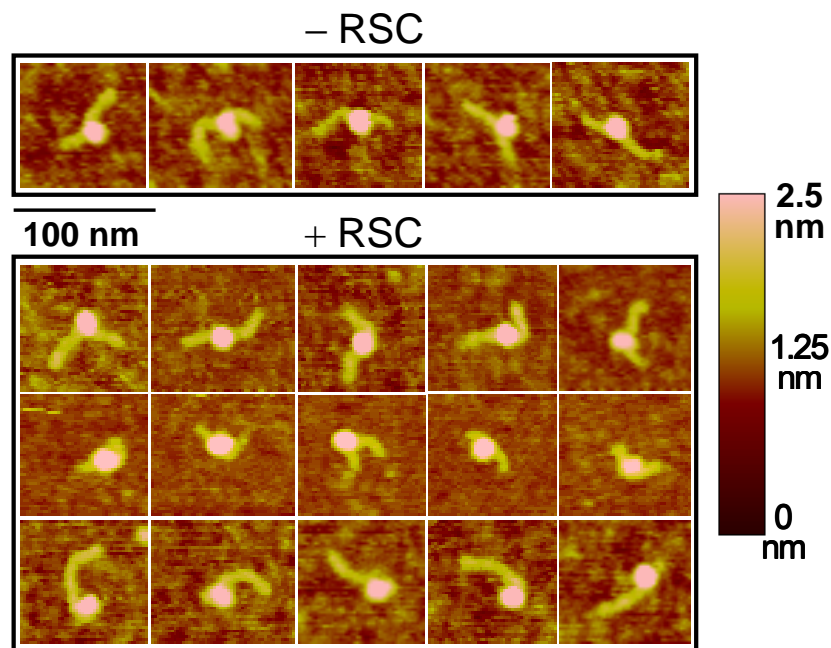
Figure II.1. **AFM visualization of RSC mobilized nucleosomes.** AFM topography images of centrally positioned nucleosomes reconstituted on 255 bp 601 positioning sequence and incubated for 30 minutes with ATP at 29°C in the absence of RSC (first row) or in the presence of RSC (2nd, 3rd and 4th rows). In the absence of RSC only centrally positioned "standard" nucleosomes are observed, while in the presence of RSC three types of nucleosomes were detected, "standard" centrally positioned nucleosomes (second row), nucleosomes with shorter "arms" (third row) and slided end-positioned nucleosomes (fourth row).

To further study how the different groups of particles were generated we have carried out remodeling reactions with two different amounts of RSC (30 and 60 fmol) and separated the reaction mixtures on PAGE under native conditions (see schematics in Figure II.2A) (Note that even at the higher amount of RSC used in the remodeling reaction it was at subsaturating concentration relative to the nucleosomes, i.e. roughly 10 times less RSC per nucleosome). Then the upper and the lower nucleosome bands were excised from the gel, the nucleosomes were eluted from the gel slices and visualized by AFM (Figure II.2B-E). The control sample (incubated with ATP in the absence of RSC and consisting of a single upper band) contained, as expected, only centrally positioned nucleosomes (see inset of Figure II.2B).

In contrast, the particles isolated from the upper band of the samples incubated with RSC were either identical to the controls or exhibited short free DNA arms (see insets in Figure II.2C). The frequency of nucleosomes with short arms dramatically increased when a higher amount of RSC was used in the remodeling reaction (Figure II.2D, inset). The lower band contained mainly completely mobilized nucleosomes (inset in Figure II.2E).
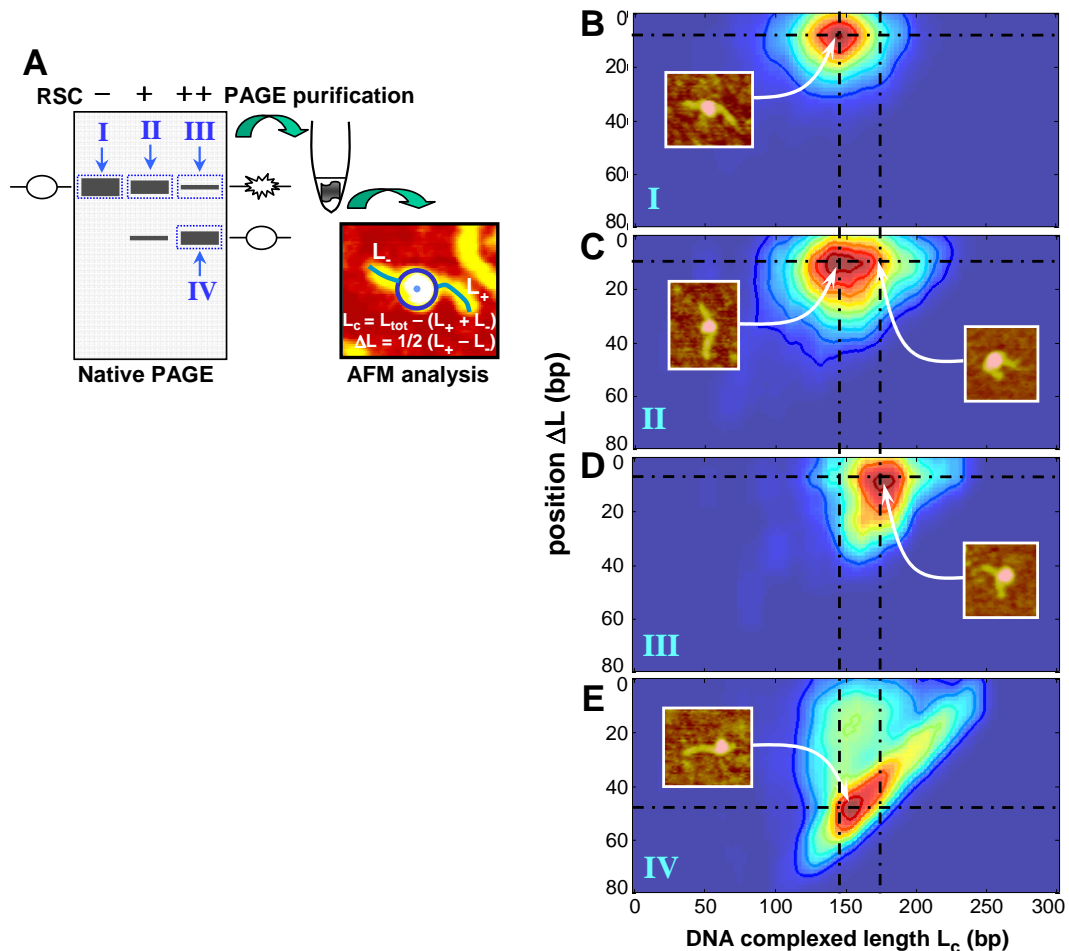
Figure II.2. The initial step of the RSC nucleosome mobilization reaction is the generation of a stable, non-mobilized particle containing 180-190 bp of histone octamer associated DNA. (A) Schematics of the experiment. Centrally positioned nucleosomes, reconstituted on 255 bp 601 positioning sequence, were incubated in the presence of ATP for 30 minutes at 30°C in either the absence (-) or the presence of 30 fmol (+) or 60 fmol (++) of RSC. After arresting the reaction, the mixtures were run on a 5% PAGE under native conditions. Then both the upper and the lower nucleosomal bands were excised from the gel, the nucleosomes were eluted and visualized with AFM. The different gel eluted particles (fractions I, II, III and IV) were indicated by arrows. The lower right part of the figure illustrates the schematics of the measurement of histone octamer DNA complexed length L_c and the position ΔL of the nucleosome relative to the center of the DNA sequence. Dark blue line: contour of the nucleosome. Light blue point: centroïd of the histone octamer. Blue dot circle: excluded area of the histone octamer. Light blue line: skeletons of the free DNA arms. Color scale: from 0 to 1.5 nm. The color indicates the probability to find a nucleosome with the DNA complexed length L_c and the position ΔL. Blue corresponds to a low probability and red to a high probability. **(B)** 2D histogram $L_c/\Delta L$ representing the DNA complexed length L_c along with the nucleosome position ΔL (N = 1254 nucleosomes) for nucleosomes incubated in absence of RSC (control) under the

conditions described in (A) and gel eluted (fraction I, see (A). (**C**) and (**D**), 2D histograms for the upper gel band eluted nucleosomes incubated with 30 fmol (fraction II, see (A), N=635 nucleosomes and 60 fmol, (fraction III, see (A) N=255 nucleosomes, of RSC. (**E**) 2D histogram for the nucleosomes eluted from the excised lower gel band after incubation for 30 minutes with RSC (N= 538 nucleosomes). The inserts show the distinct nucleosome species corresponding to the different regions of the 2D histograms.

The clear visualization of the free DNA arms allows the precise measurement of the DNA length of each arm (indicated as L_+ and L_- for the longer and the shorter arm, respectively). To measure the length of each arm, we have excluded the octamer part and the trajectory of the free DNA was determined by using morphological tools avoiding false skeletonization by heuristic algorithm (Figure II.2A and Materials and Methods). The precise measurements of the length of the arms allowed the calculation of both the length of the DNA complexed with the histone octamer L_c ($L_c = L_{tot} - L_+ - L_-$, where L_{tot}= 255 bp is the length of the 601 fragment used for reconstitution) and the position of the nucleosome relative DNA template center ΔL= ($L_+ - L_-$)/2. The 2D histogram $L_c/\Delta L$ for the control nucleosomes (treated with ATP in the absence of RSC and eluted from the gel particles) is presented in Figure II.2B. The maximum of the distribution peaked at ~ 145 bp and ΔL is ~5-8 bp, which is in a good qualitative agreement with the determination of the nucleosome position by biochemical approaches. Importantly, in the absence of ATP, RSC has no effect on the $L_c/\Delta L$ map (data not shown)

The 2D histograms $L_c/\Delta L$ for the nucleosomes incubated with RSC (in the presence of ATP) and eluted from the gel slice nucleosomes were, however, quite different (Figure II.2 C-E). The data show that both variables, Lc and ΔL, are significantly different in the distinct RSC generated nucleosome populations. Indeed, at the lower amount (30 fmol) of RSC present in the remodeling reaction the $L_c/\Delta L$ map for the nucleosomes isolated from the upper electrophoretic band was getting wider indicating that particles with overcomplexed DNA (more than 150 bp in length) were generated (Figure II.2C). The presence of the higher amount (60 fmol) of RSC resulted in the generation of mainly particles with short free DNA arms (isolated from the upper band) and containing about 180-190 bp DNA in complex with the histone octamer (Figure II.2D). Importantly, the nucleosome position ΔL relative to the DNA ends in these particles remained essentially the same as in the control particles, suggesting that the increased amount of DNA associated with the octamer is achieved through pumping of about 15-20 bp of DNA from each free DNA arm without nucleosome repositioning. For simplicity, further in the text we will call these particles *remosomes* (remodeled nucleosomes).

The Lc/ΔL map for the particles, eluted from the lower electrophoretic band of the RSC incubated samples in the presence of ATP, showed that both the complexed DNA length L_c and their position ΔL have altered and had average values of Lc ~150 bp and ΔL~50 bp. Thus, they represented a population of nucleosomes relocalized to the DNA end.

II.3.2 The remosomes are ensemble of distinct structures with different DNA conformation

The AFM visualization of the RSC remodeling reaction products gave an intriguing insight into their organization. The AFM experiments could be, however, affected by the deposition of the samples on the functionalized mica surface. To overcome this potential problem the RSC remodeling reaction products were also visualized by Electron Cryo-Microscopy (EC-M). Indeed, EC-M experiments, carried out in vitrified solution without any fixation and use of contrasting reagents, provide high resolution images of the "native" 3D structure of the studied material. EC-M has very successfully been used to investigate the structure of different chromatin samples, including isolated nucleosomes and 30 nm chromatin fibers (Bednar et al., 1995; Bednar and Woodcock, 1999). The EC-M pictures of the RSC reaction products clearly show, as in the case of AFM images, the presence of three different types of structures, namely unperturbed centrally positioned nucleosomes, end-positioned nucleosomes and remosome-like structures (Figure II.3A). Typically, the remosomes exhibited shorter free DNA arms. Importantly, the DNA conformation of each individual remosome was distinct and irregular and differed from the round shaped DNA conformation of the centrally positioned or slided end-positioned particles (Figure II.3A). These results are in complete agreement with the AFM data (compare Figure II.3A with Figure II.1) and demonstrate that the remosomes do not exhibit a single, well defined organization but instead represent an ensemble of different nucleosome-like particles with distinct trajectories of an extended associated DNA.

The described above results were obtained by using nucleosomes reconstituted on 601 DNA sequences. The 601 sequence is, however, an "artificial" sequence, which was not so far identified in the studied genomes. Then the question arises whether the described remosome structures could be generated when using natural DNA sequences for nucleosome reconstitution. To test this we have studied the remodeling of nucleosomes reconstituted on a 255 bp DNA fragment, containing the 5S RNA gene of *Xenopus borealis* (Figure II.3B). Under our conditions of reconstitution the majority of the nucleosomes were centrally located

(Figure II.3B, the two first pictures of the 1st row). Some amount of end-positioned nucleosomes was also observed, which reflects the weaker positioning signal of the 5S DNA. In both cases the nucleosomes exhibit well defined round shape and relatively long free DNA arms (Figure II.3B, 1st and 2nd rows). Upon incubation with RSC, as expected, the amount of the centrally positioned nucleosomes strongly decreases while that of end-positioned nucleosomes increases (results not shown). Importantly, remosome-like structures with larger dimensions, irregular shape and shorter free DNA arms were observed (Figure II.3C, 3rd and 4th rows). We conclude that RSC has the capacity to generate remosomes on natural DNA sequences.

We have also studied the RSC remodeling of trinucleosomes, reconstituted on a DNA fragment, containing three 601 sequences. The individual nucleosomes within the trinucleosomes showed a well defined round shape and are equally spaced (Figure II.3C, 1st row). Incubation of these templates with RSC (in the presence of ATP) resulted either in nucleosome sliding and consequently in closely spaced nucleosomes within the trinucleosomes (Figure II.3C, 4th row) or in the generation of remodeled templates (Figure II.3C, 2nd and 3rd rows), where one of the nucleosomes exhibits remosome-like conformation with larger and irregular shape. No such remodeled trinucleosomes were observed upon incubation with RSC, but in the absence of ATP. These data illustrate the capacity of RSC to generate remosomes within nucleosomal arrays.

Since a single nucleosome can be converted into a remosome within the trinucleosomal array, this suggests that RSC is associated with a single nucleosome within the array and that it remodels only one nucleosome at a time. To study the association of RSC with the trinucleosomes, H1-depleted trinucleosomes were isolated from chicken erythrocyte nuclei and complexed with RSC. Then they were fixed with formaldehyde, negatively stained and used for the EM experiments. Note that under the conditions used in the AFM and EC-M experiments, we were able to observe only very few RSC-nucleosome complexes, suggesting that once the remosomes are formed or the nucleosomes are mobilized, RSC dissociates from its substrate. Fixation was, thus, required to visualize the RSC-nucleosome complex under our experimental conditions.
The RSC alone showed the typical "crescent" shape conformation with a central cavity (Figure II.3D, 1st row), a result in agreement with the previous reports (Leschziner et al., 2007; Asturias et al., 2002). However, when RSC was allowed to associate with the

trinucleosome, a much larger structure than a single nucleosome was observed (Figure II.3D, compare the structures of the trinucleosomes of the 2nd row with those of the 3rd row). The linker DNA connecting this large structure with the adjacent nucleosomes was clearly visible (Figure II.3D, 3rd row). We attributed this structure to the RSC-single nucleosome complex. Interestingly, this large structure exhibited a uniform staining, demonstrating that the nucleosome indeed filled the RSC cavity (Figure II.3D, 3rd row). This result is in agreement with the recent cryo-EM data showing that RSC forms a complex with a single isolated nucleosome (Chaban et al., 2008) and further illustrates that this is also the case when nucleosomal arrays are used as substrate for the remodeler.
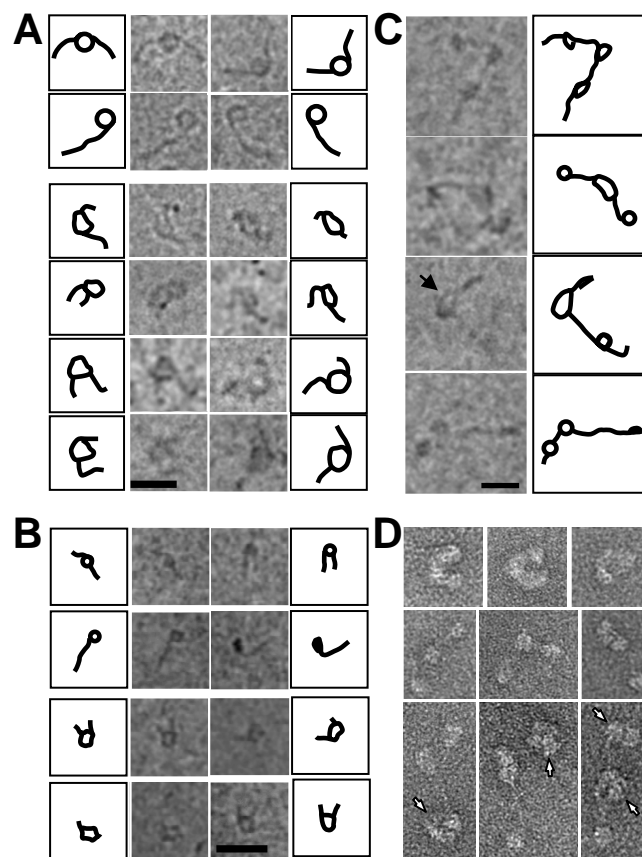
Figure II.3. **Electron Cryo-Microscopy (EC-M) of the RSC treated mono- and trinucleosomes shows that different species are present in the RSC remodeling reaction.** (**A**) Centrally positioned nucleosomes reconstituted on a 255 bp 601 DNA were treated with RSC for 30 minutes at 29°C in the presence of ATP (under these conditions ~ 30% of the nucleosomes were completely mobilized) and then immediately vitrified. The first two rows show the nucleosomes exhibiting 'standard' structure, i.e. non-mobilized nucleosomes (the first row) and completely mobilized nucleosomes (the second row). The remaining four rows show the EC-M micrographs of the nucleosomes with altered structure. Each micrograph is accompanied by schematic drawing illustrating the shape of the DNA observed in the micrographs. (**B**) Incubation of 5S nucleosomes with RSC results in the generation of remosomes. Centrally positioned nucleosomes were reconstituted on a 255 bp DNA fragment containing the 5S somatic gene of *Xenopus borealis*. The 5S nucleosomes were treated with RSC as described in (A), vitrified and visualized by cryo-EM. Non-affected (first row) and end-mobilized (second row) by

RSC particles as well as RSC-generated remosomes (third and fourth rows) are shown. (**C**) RSC has the capacity to generate remosomes in nucleosomal arrays. Trinucleosomes were reconstituted on a DNA fragment consisting of three 601 repeats. The length of each repeat was 197 bp. The trinucleosomes (containing a centrally positioned nucleosome within each 601 repeat) were treated with RSC as described in (A) and then immediately vitrified. The first row illustrates the structure of a trinucleosome un-affected by RSC. The second and the third rows show a typical structure of trinucleosome, containing a remosome (the black arrow indicates the centrally located remosome within the trinucleosome). Note the altered structure of the remosome compared to the end-positioned nucleosome in the trinucleosome. The fourth row shows a trinucleosme in which the centrally positioned nucleosome has been mobilized. Each micrograph is accompanied by schematic drawing illustrating the shape of the DNA observed in the micrographs. (**D**) The RSC complex is associated with a single nucleosome within a trinucleosome. Native H1-depleted trinucleosomes were incubated with RSC and the RSC-trinucleosome complexes were fixed by 0.1 % formaldehyde. The material was then negatively stained and visualized by conventional EM. The first and the second row show representative electron micrographs of RSC and trinucleosomes alone, respectively. On the third row are shown the RSC-trinucleosome complexes. Note that RSC is associated with a single nucleosome (see arrows). (Scale bar 50nm)

II.3.3 The "in gel one pot assay" shows highly perturbed histone-DNA interactions within the remosome

To biochemically characterize the DNA path within the remosome at higher resolution we have developed a novel method based on the recently reported "one-pot" assay for the accessibility of DNA towards restriction enzymes in the nucleosome core particle (Wu and Travers, 2004). We called this method "in gel one pot assay" (see the schematics of the method in Figure II.4A). Briefly, we have used eight different mutated 255 bp 601.2 DNA sequences. Each one of the sequences bears a *Hae*III restriction site (designated dyad-0 (d_0) to dyad-7 (d_7), where the number indicates the number of helical turns from the dyad). Each restriction site has the same rotational position with an outward-facing minor groove (Wu and Travers, 2004). With this system it is possible to measure the accessibility of the nucleosomal DNA at many different sites in a single reaction and any change in the rotational position or protection of the site (s) could be readily detected.

We have produced the above described eight 601.2 sequences by PCR amplification by using ^{32}P-end labeled primers and then we used them for reconstitution of centrally positioned nucleosomes. An equimolar mixture of the eight centrally positioned nucleosomes was incubated with RSC in the presence of ATP in a way to produce about 50% (relative to the total initial amount of nucleosomes) of slided end-positioned nucleosomes and the reaction mixture was run on a 5% PAGE under native conditions (Figure II.4A). Then the upper band (containing the non-slided particles) was excised and digested in gel with increasing amount

of *Hae*III under appropriate conditions. DNA fragments were isolated from the in gel *Hae*III digested nucleosome particles and separated on 8% sequencing PAGE. The same experiment was carried out with control (incubated with RSC but in the absence of ATP) nucleosomes. After exposure of the dried gel, product bands from the experiment were quantified and expressed as percentage of cut fraction.

A typical experiment is presented in Figure II.4B and C. In the absence of ATP, the accessibility of dyad-7 to *Hae*III differed from that of the other dyads. Indeed, even at low concentration (0.125 u /μl) of *Hae*III used ~30% of dyad-7 was cleaved (Figure II.4B and C). Increasing the concentration of the restriction enzyme resulted in an increased dyad-7 cleavage, which reaches ~70-75 % at 8 u/μl *Hae*III. An apparent increase of the accessibility was also observed for dyad-6, which reached 20-25% cleavage at the highest concentration (8 u/μl) of *Hae*III. The cleavage at all the other sites was very low and remained largely unchanged at all concentrations of *Hae*III, suggesting a weak accessibility of these sites. These results are in complete agreement with the previously reported data and are consistent with a transient unwrapping of DNA between dyads-7 and -5 (Wu and Travers, 2004). The picture was, however, completely different for the remosome fraction. In this latter case, the accessibility of dyad-7 sharply decreased upon increasing the concentration of the enzyme (down to ~ threefold decrease at the highest concentration 8 u/μl *Hae*III). The accessibility of all the other sites (from dyad-6 to dyad-0) dramatically increased, the most pronounced increase (up to 10-15 fold in the different experiments) being observed at dyad-0. These data demonstrate that within the RSC generated remosome the DNA organization differed substantially from that of the unremodeled particle. The decrease accessibility at dyad-7 would reflect the RSC "pumping" of 15-20 bp free linker DNA and the association of the sites around dyad-7 with the histone octamer and respectively protection of these sites against *Hae*III digestion. The increased accessibility in the remosome of all the remaining dyads could be viewed as an evidence for strong perturbations in the histone-DNA interactions at these internally located sites within the remosome. Note that the efficiency of *HaeIII* cleavage along the nucleosomal DNA was not completely uniform, but instead displayed a parabolic-like shape (see Figure II.4C) with highest values at d_0 and d_7. Since within the native nucleosome the strongest histone-DNA interactions are found around d_0, (Luger et al., 1997) this shows that RSC has specifically altered these interactions and suggests that this alteration

of the histone DNA-interactions around d_0 is important for further mobilization of the remosomes.
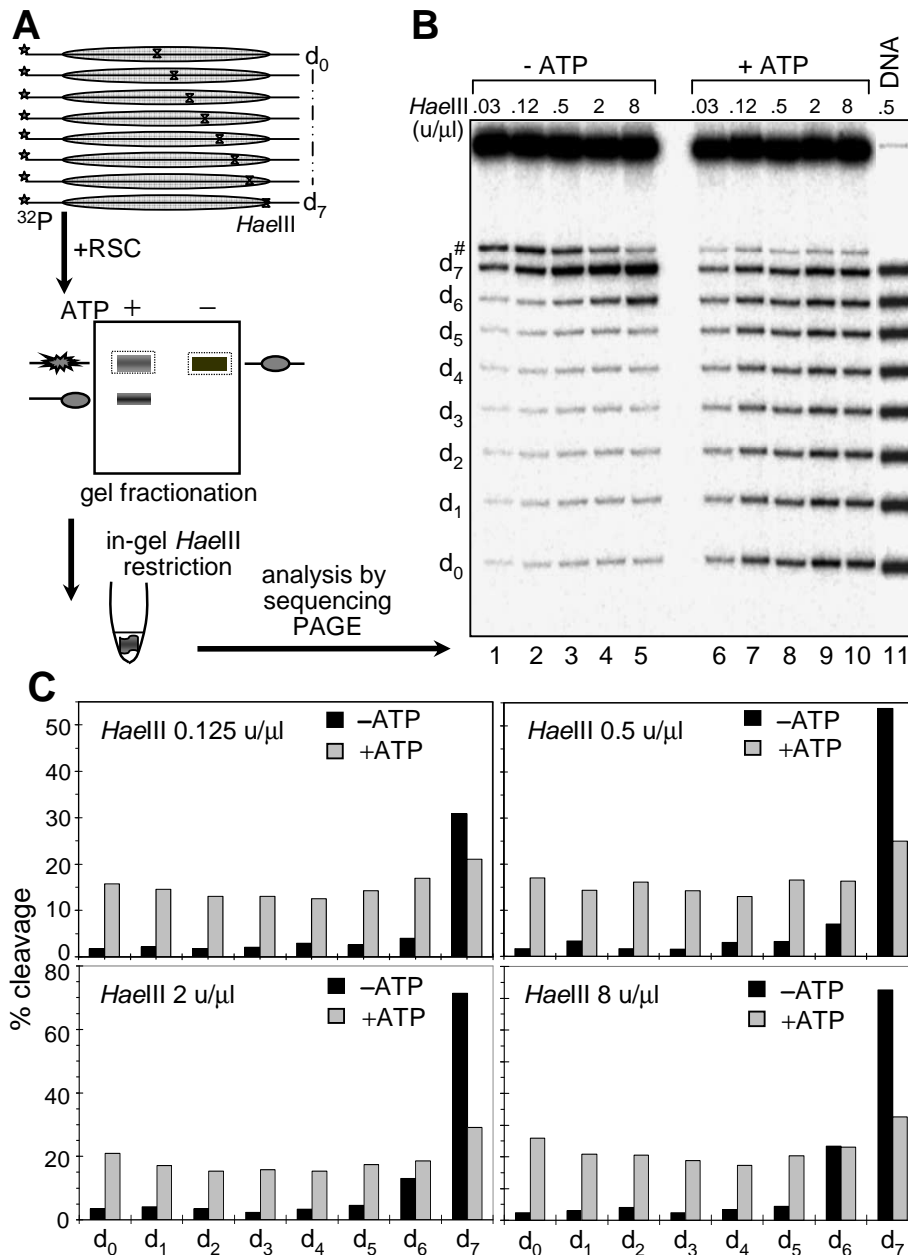
Figure II.4. In gel "one pot" restriction accessibility assay of the RSC generated remosomes. (**A**) Schematics of the in gel "one pot" assay. (**B**) *Hae* III DNA digestion pattern of the non-slided nucleosomes incubated with RSC in the absence (left panel) or presence (right panel) of ATP. The excised gel slice containing the control (incubated in the absence of ATP) or the non-mobilized (but treated with RSC in the presence of ATP) nucleosomes were incubated with the indicated units of *Hae*III for 5 minutes at 29°C. DNA was then isolated and run on an 8% sequencing PAGE. Lane 11, naked DNA digested 0.5 U/μl *Hae* III. The # indicates a fragment which corresponds to a *Hae* III site present only in "dyad 7" 601.2 fragment and located at 4 bp from the dyad 7 (d_7) site (**C**) Quantification of the data presented in (B).

II.3.4 The remosomes are intermediate structures generated during the RSC nucleosome mobilization process.

All the above data strongly suggest that the remosomes are intermediate structures generated by RSC that are further mobilized and converted completely into end-positioned nucleosomes. To further confirm this we have designed an experiment, which allowed the measurement of the amount of the various nucleosome species present at different stages of the remodeling reaction (Figure II.5). The protocol for these experiments is presented in Figure II.5A. Centrally positioned nucleosomes were incubated with RSC either in the presence or absence of ATP for time points ranging from 0 to 64 minutes. After arresting the reaction they were submitted to partial DNase I digestion and run on PAGE under native conditions. Then the fractions containing the remosomes (the electrophoretic band with lower mobility) and two of the slided fractions (obtained after 48 and 64 minutes of incubation with RSC, respectively) were excised from the gel, the DNA was eluted and run on a sequencing gel (Figure II.5B). Upon increasing the time of incubation with RSC the accessibility of DNA within the remosome fractions was strongly altered (lanes 2-8) and in contrast to the digestion pattern of the control nucleosomes (incubated with RSC in the absence of ATP, lane 1) becomes very similar to naked DNA (lane DNA) and that of the slided nucleosomes (lanes 9, 10). Since no mobilization of the histone octamer was observed in the remosome fraction (see Figure II.2), we attributed the altered DNase I digestion pattern to reflect strong perturbations of the histone-DNA interactions within the remosome, a result in complete agreement with the data of "one pot in gel assay " (Figure II.4).

As the RSC remodeling reaction proceeds, the alterations in the DNase I patterns of the fractions containing the remosomes are characterized by the disappearance or decrease of intensity of some specific for the nucleosome bands and the appearance (or increase of intensity) of some bands specific for naked DNA (Figure II.5B, see bands indicated by asterisks). We have used this effect to measure the part of intact nucleosomes in the remosome fraction (see Materials & Methods section for detail). The part of the slided nucleosomes was directly measured from the native PAGE (Figure II.5A). Since the total amount of all type of nucleosomes in the RSC reaction was known, this has allowed the calculation of the part of remosomes present in the reaction mixture (Figure II.5C).

As seen, during the remodeling reaction, the amount of intact nucleosomes rapidly decreases, while that of the slided nucleosomes increases, but with lower rate (Figure II.5C, compare the initial slope of the "intact" nucleosome curve with that of the "slided" nucleosome curve). Consequently, at the initial times of the remodeling reaction the amount of remosomes increases, reaches a plateau, which is followed by its gradual decrease as the remodeling reaction proceeds (Figure II.5C). Note that the initial rate of remosome formation is higher than that of slided nucleosomes (Figure II.6C, compare the initial slope of the "remosome" curve with that of the "slided" nucleosome curve). Importantly, when using our AFM data to measure the proportion of each individual particle species in the RSC reaction mixture very similar curves were obtained (See supplementary Figure II.S2). Therefore, the use of two completely independent techniques has led to the same results. This demonstrates that indeed the remosomes are intermediate products generated by RSC in ATP-dependent manner, which are further converted into slided, end-positioned particles.
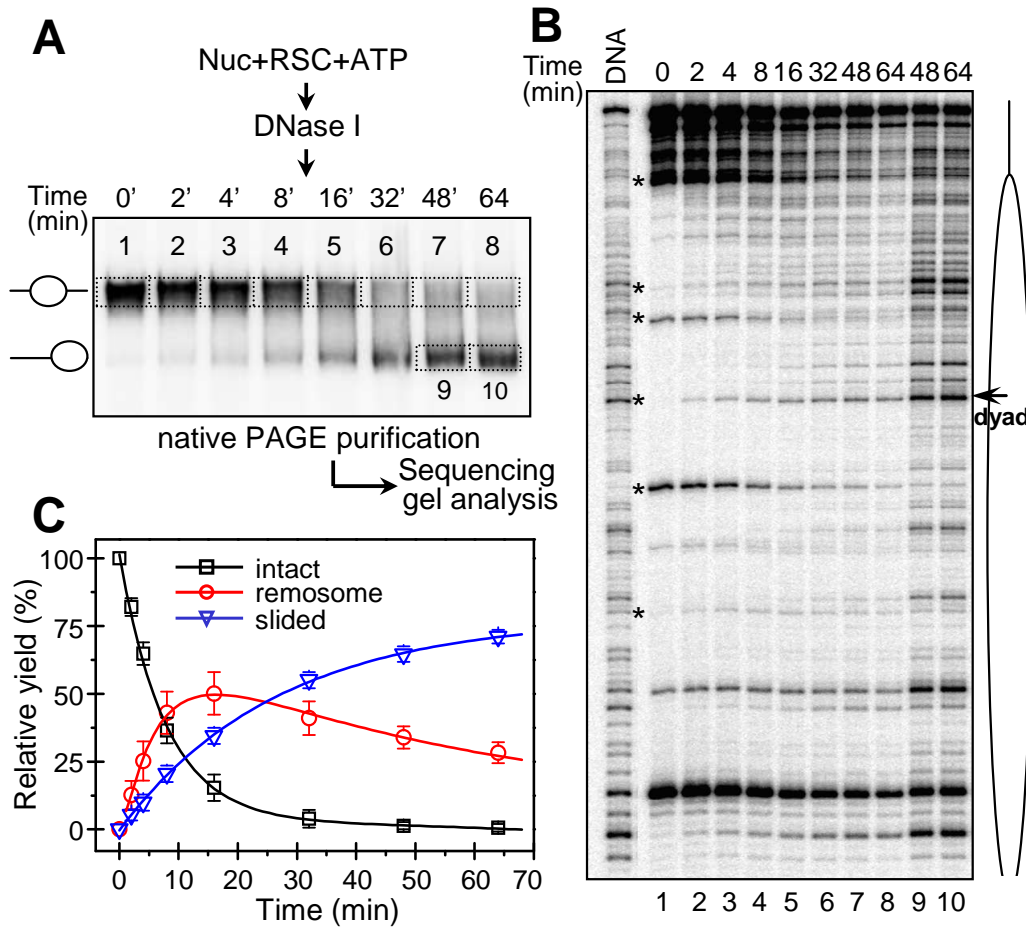
Figure II.5. The remosomes are intermediate structures generated during the RSC mobilization reaction. (**A**) Schematics of the experiment. Centrally positioned nucleosomes were treated with RSC (in the presence of ATP) for the times indicated (ranging from 0 to 64 minutes) and after arresting the reaction they were incubated with DNase I and they were separated on a native PAGE. Then the upper bands (from 1 to 8) and the lower bands 9 and 10 (obtained upon incubation with RSC for 48 and 64 minutes, respectively) were cut from the gel and the nucleosomal particles were eluted. DNA was isolated from the different eluted samples and run on DNA sequencing gel. The changes in the intensity of the DNA bands (in the DNase I digestion pattern) specific either for the nucleosome or free DNA (marked with asterisk in panel B) were used to quantify the fraction of intact nucleosomes present at a given time in the remodeling reaction mixture (see Material and Method section for detail). The amount of mobilized nucleosome was directly measured from the native PAGE (see panel A). The fraction of remosomes present in the remodeling reaction mixture at a given incubation time was calculated as: %(remosome) = 1- %(intact nucleosomes) – %(slided nucleosomes). (**B**) 8% sequencing PAGE of the isolated DNA from the RSC remodeled and DNase I digested particles. At the bottom of the gel are indicated the numbers of the different fractions presented in panel (A). At the top of the gel are indicated the times of incubation with RSC. The last two lanes (48 and 64 minutes) show the DNase I digestion pattern of the gel purified mobilized particles (see panel A). DNA, DNase I digestion profile of free 601 DNA. At the right part of the figure is presented schematically the position of the nucleosome; the arrow shows the location of the nucleosome dyad. Bands, which change in intensity (indicated with asterisk), were used for calculation of the fraction of intact nucleosomes remaining in each remodeling reaction. (**C**) Normalized fractions of intact nucleosomes, remosomes and slided nucleosomes (relative yields) determined from A and B versus the reaction time. Note that upon incubation with RSC an initial rapid increase of the amount of remosomes is observed, then it reaches a plateau, which is next followed by its gradual decrease as the remodeling reaction proceeds.

101

II.3.5 The remosomes are *bona fide* substrates for mobilization by RSC

If the remosomes are intermediates of the RSC nucleosome mobilization reaction, they should be efficiently mobilized by RSC. We have addressed this question by using gel purified remosome fractions. Briefly, we have incubated with RSC (in the presence of ATP and under the same conditions as in Figure II.5) centrally positioned 601 nucleosomes for 16 and 48 minutes and after arresting the reaction we have separated the different species on native electrophoresis (Figure II.6A). Then we have cut the gel slices containing the remosome fractions (R and R+, obtained after 16 and 48 minutes of incubation, respectively), the slided nucleosomes (S), as well as the control fraction (N) (Figure II.6A). Note that under these conditions of incubation with RSC, both fractions (R and R+) contained mainly remosomes (see Figure II.5C). The particles from R, R+, S and N fractions were eluted from the gel and a RSC mobilization assay was carried out in the presence of ATP (Figure II.6B). As seen, the remosome fractions (R and R+) as well as the control nucleosomes (N) were efficiently mobilized by RSC, while the slided fraction, as expected, was not affected. In the absence of ATP, no one of the different nucleosome species was mobilized (results not shown). We conclude that the remosomes are good substrates for RSC, which can be mobilized by the remodeler in a ATP-dependent manner.
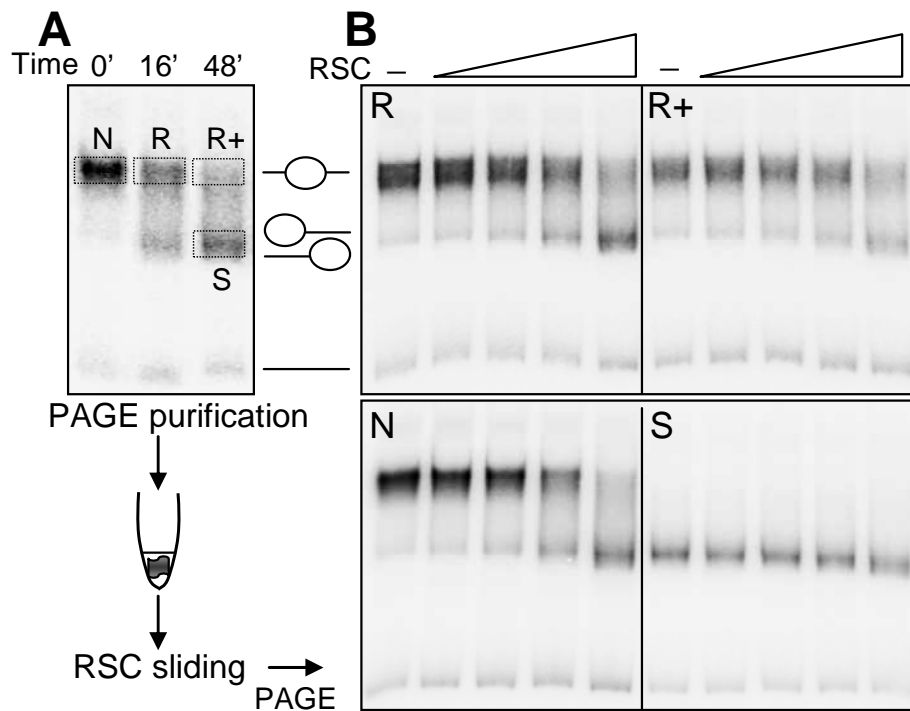
Figure II.6. The remosomes are *bona fide* substrates for RSC. (A) Schematics of the remosome mobilization experiment. Centrally positioned 601 nucleosomes were treated (under the same conditions as in Figure II.5) with RSC in the presence of ATP for the times indicated. The reaction was arrested and the reaction mixtures were loaded on native PAGE. After separation of the different nucleosome species, the bands containing the remosomes (R and R+), the slided nucleosomes (S) and the control nucleosomes (N) were excised from the gel and the particles were eluted. Then they were incubated again with increasing amount of RSC and the RSC-induced particle mobilization was visualized by using native PAGE. The different nucleosome species are indicated on the right part of the panel. **(B)** Mobilization of the remosome fractions R and R+, the control nucleosomes (N) and the slided end-positioned nucleosomes (S). Note that both remosome fractions R and R+, in contrast to the end-positioned nucleosomes (S), are mobilized by RSC.

II.4 Discussion

In this work we have studied the mechanism of nucleosome mobilization by the remodeling assembly RSC. Reconstituted centrally positioned nucleosomes flanked by two free DNA arms were incubated with RSC and the products of the reaction were visualized by AFM, EM and EC-M. EM was also used to analyze the complex of RSC with tri-nucleosomal templates. Our results, in agreement with the recently reported data (Chaban et al., 2008), demonstrate that RSC is associated with a single nucleosome suggesting that it remodels only one nucleosome at a time. We show that as a result of the remodeling reaction two types of

products were generated: nucleosome-like particles (remosomes) containing 180-190 bp DNA and mobilized particles with the histone octamer located at either one of the DNA ends. RSC has also the capacity to generate remosomes in short nucleosomal arrays. Remosomes are stable particles that can be separated from the slided end-positioned nucleosomes by PAGE under native conditions and eluted from the gel. Both free DNA arms of the remosomes are shorter compared to those of the non-remodeled particles and the position of the histone octamer relative to the center of the DNA fragment remains identical to that of the non-remodeled structures. EC-M visualization demonstrated that the DNA wrapping around the histone octamer of the individual remosomes was distinct but quite irregular, and importantly strongly differed from the helical projection of the DNA path of both the non-remodeled or slided end-positioned particles. The histone-DNA interactions within the remosome were strongly perturbed as shown by both the novel "in gel one pot assay" method and DNase I footprinting. These data, taken together, allow the conclusion that the remosomes do not exhibit a single, well defined organization, but instead represent a multitude of structures, each structure exhibiting a distinct DNA trajectory around the associated histone octamer. The AFM visualization of the products of the remodeling reaction carried out at different concentrations of RSC strongly suggests that the remosomes are intermediate structures in the mobilization process, which are subsequently converted into normal, but end-positioned nucleosomes. This claim was supported by the experiments demonstrating the evolution of the different nucleosome species during the mobilization process and the capacity of RSC to efficiently mobilize the remosomes.

Based on our and previous data we propose the following model for the mechanism of RSC nucleosome remodeling (see Figure II.7). A single RSC complex associates with a single particle when using mononucleosomal (Leschziner et al., 2007; Chaban et al., 2008) or polynucleosomal template (Figure II.3). This nucleosome "fills" the cavity of RSC with its dyad axis accessible from the solution as suggested (Leschziner et al., 2007; Chaban et al., 2008). It utilizes a two-step mechanism to mobilize the nuclesome (Figure II.7). By using the energy of ATP hydrolysis, RSC pumps 15-20 bp DNA from the each one of the free DNA linkers without repositioning of the histone octamer (the AFM data). This has two major consequences: (i) creation of a 30-40 bp loop (or bulge) in the vicinity of the dyad and thus, disruption of the strongest histone-DNA interaction within the nucleosome and, (ii) changes in the DNA path within the nucleosome. The particle created in this way no longer fits in the RSC cavity and the remodeler dissociates from the nucleosome. The loop is, however,

unstable, it propagates and stops at different sites along the nucleosomal DNA, where it partially spreads. Since the pumped additional 15-20 bp DNA of each linker is found associated with the histone octamer (the "in gel one pot assay" results), the spread loop cannot dissipate. As a result, a multitude of stable structures with distinct, irregular DNA path is generated, i.e. the remosome is formed. During the second step of the reaction, RSC functions as a true translocase, by pumping and releasing DNA as it has been suggested by single molecule experiments (Lia et al., 2006; Zhang et al., 2006). To fulfill its translocase activity, RSC has, however, to change its conformation in order to properly interact with the remosomes and to translocate DNA.
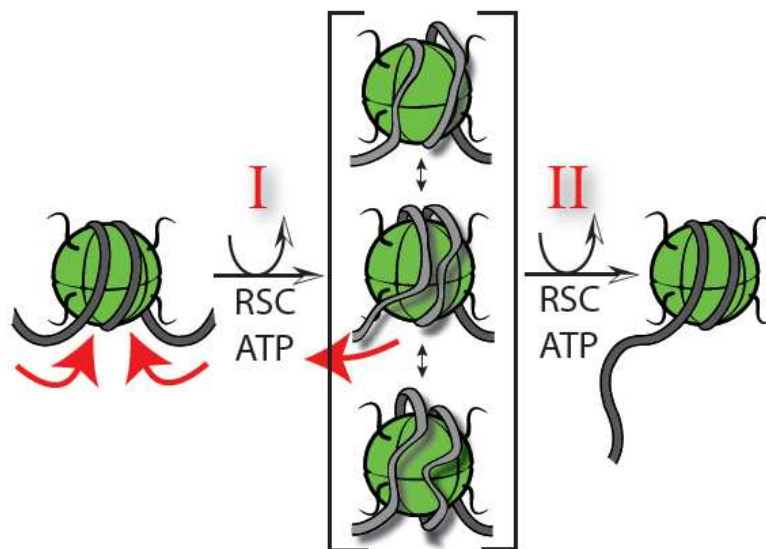
Figure II.7. Schematic representation of the two step RSC-induced nucleosome mobilization. In a first step (I) ATP hydrolysis is used by RSC to remodel a middle positioned nucleosome by pumping ~15-20 bp from both sides. The resulting remosomes can exhibit various configuration of their over-complexed DNA. In a second step (II), ATP hydrolysis by RSC results into the translocation of the DNA to produce an end-positioned nucleosome.

The proposed model indirectly implies that the translocation of DNA is performed through the remosome, a claim which is in complete agreement with the experimental data showing that the remosomes are efficiently mobilized by RSC.

Earlier reports have suggested that chromatin remodeling machines from the SWI2/SNF2 family are able to generate stable remodeled nucleosomes in which the DNA-histone interactions are altered (Fan et al., 2003; Fan et al., 2004; Narlikar et al., 2002). In this study we have for the first time directly demonstrated the existence of such particles (remosomes) and have both visualized the path of the DNA in remosomes and also importantly have

distinguished these particles from mobilized nucleosomes. We conclude that the remosome contains up to ~40 bp more DNA than the initial unremodeled core particle. This observation shows that remodeling by RSC proceeds initially by the formation of a bulge or loop rather than by a twist propagation mechanism. In formal terms the rotational tracking of the RSC complex around the sugar-phosphate backbone is manifested principally as a change in writhe of the octamer-associated DNA. Further the location of disrupted contacts in the vicinity of the dyad indicates that 'loop' propagation does not proceed from one of the outer extremities of the wrapped DNA. Rather this central position is consistent with the facilitation of remodeling by HMGB proteins (Bonaldi et al., 2002) which also can increase the accessibility of octamer-bound DNA at the dyad (Ragab and Travers, 2003). We speculate that the major *in vivo* function of RSC is the generation of remosomes. Since this process would minimize nucleosome collision, it would in principle facilitate several vital nuclear processes including both DNA repair and transcription factor binding.

II.5 Experimental Procedures

II.5.1 Preparation of DNA fragments

The 255 bp 601 DNA probe used for reconstitution of centrally positioned nucleosomes was PCR amplified from pGEM-3Z-601.1 plasmid (kindly provided by J. Widom). 5' end labeling was performed by using ^{32}P-labeled primer in PCR. For 'One Pot Restriction enzyme Assay' a set of eight pGEM-3Z-601.2 mutants were utilized, each containing *HaeIII* site at a different superhelical location, as described before (Wu and Travers, 2004; note that the "dyad 7" fragment contains an additional *HaeIII* site located at 4 bp away from the d7 site). Briefly, a 281 bp fragment was amplified using primers targeting the vector specific sequence flanking the 601.2 sequence. Labeling of the fragment was done as described above. The fragments were subsequently digested with *Sph*I to get a fragment of 255 bp with 57 and 51 bp linker DNA on left and right side respectively. All the fragments were purified on 6% native acrylamide gel prior to use for nucleosome reconstitutions. Additionally, A 255 bp 5S DNA was PCR amplified from pXP-10 plasmid for Electron Cryo-Microscopy experiments to visualize nucleosome remodeling reaction products.

II.5.2 Proteins and Nucleosome reconstitutions

Recombinant *Xenopus laevis* full-length histone proteins were expressed in form of inclusion bodies in *E. coli* Strain BL21(DE3) and purified as described (Luger et al., 1999). Yeast RSC complex was purified essentially as described (Cairns et al., 1996). Nucleosome reconstitution was performed by the salt dialysis procedure (Mutskov et al., 1998). For biochemical experiments requiring ^{32}P-end labelled DNA, 100 ng of ^{32}P- labelled 255 bp 601.1 or an equimolar mixture of the eight different ^{32}P-labelled 255 bp 601.2 mutated DNA fragments (100 ng) were added to the reconstitution mixture.

II.5.3 Nucleosome remodeling reaction

Typical remodeling reactions were performed with 150 fmol of nucleosomes and ~15 fmol of RSC in remodeling buffer (RB) 10 mM Tris pH 7.4, 5% glycerol, 1 mM rATP, 2.5 mM MgCl$_2$, 1 mM DTT, 100 µg/ml BSA, 50 mM NaCl, 0.01% NP40) in a volume of 7.5 µl at 29° C. In scaled up remodeling experiments nucleosome to RSC concentration ratio (~10:1) was maintained if not mentioned otherwise. It is to note that under our experimental conditions this nucleosome to remodeler ratio was sufficient to mobilize nucleosomes to saturation in 45 minutes.

II.5.4 DNase I footprinting assay

300 fmol of nucleosomes, reconstituted on ^{32}P- end-labeled 255 bp 601.1 DNA, were incubated with 30 fmol RSC in 15 µl RB for indicated time intervals. Reactions were stopped by addition of 0.02 units of apyrase and 2 µg of plasmid DNA. In the '0 time' control reaction, apyrase was added before addition of RSC. All the reactions were divided into two equal parts. In the first part, DNase I digestion was performed by addition of 0.5 units of Dnase I. EDTA was added to 25 mM to stop the DNase I cleavage. Both the undigested and DNase I digested samples were resolved in parallel on separate native polyacrylamide gels (29:1) in 0.25X TBE at room temperature. The native gel corresponding to undigested sample was used for quantitation of nucleosome sliding. From the second gel, done for resolving Dnase I digested samples, bands corresponding unmobilized and mobilized nucleosomes were

excised. DNA was eluted, filtered, deproteinized through phenol:chloroform treatment, precipitated and run on 8% denaturing PAGE.

The gel bands (Figure II.5) were quantified by integration of rectangles using the Multi Gauge v3.0 (Fuji) software. In the case of figure 5A, the fraction of mobilized nucleosomes (S) was found by dividing the signal of the fast migrating band to the total radioactivity, i.e. to the sum of the signals of the slow and fast migrating bands. The quantification of the fraction of native nucleosomes (N) present at each studied time point of the remodeling reaction (Figure II.5C) was based on the observation that upon generation of remosomes some typical nucleosomal bands disappear, while other typical naked DNA bands in the DNase I digestion profile appear concomitantly (Figure II.5B, see bands marked with asterisks). Therefore, the relative intensity of these bands is a measure of the amount of intact nucleosomes in the remodeling reaction at the respective time point. The signals of these bands for each time point of RSC remodeling, normalized to the sum of the signals of all bands (the total radioactivity in the lane) were determined by integration. These values were further normalized assuming 100% and 0% intact nucleosomes at the time points t=0 and t=64 minutes respectively. This assumption is based on the observation of a full saturation at 48 and 64 minutes of the dependencies of the intensity of each band versus time of RSC remodeling (data not shown, but see Fig II.5C, "intact nucleosomes"). Finally, values for different bands in each line were averaged and then multiplied to the corresponding fractions N+R=1-S (determined from Fig II.5A, see above). This allows the determination of the fraction of intact nucleosomes (N) present at the given time point of the remodeling reaction. The fractions of remosomes R at each time point were calculated as R=1-N-S.

II.5.5 Sliding assay on gel eluted nucleosome

Centrally positioned 150 fmole 601.1 nucleosomes were incubated with RSC in the remodeling reaction as described above. Reaction was stopped 16 and 48 minutes by addition of 0.01 units of Apyrase and 1 μg of plasmid DNA, as under these conditions the non-mobilized fraction contains essentially remodeled nucleosome particles. Reaction products were resolved on 5% native polyacrylamide gel. Bands, corresponding to unmobilized fractions from 0, 16 and 48 minute, and mobilized fraction from 48 minute reaction time points were excised. Excised bands were then cut in small pieces and soaked in 80 μl Elution Buffer (EB) containing Tris 10 mM pH7.4, 0.25 mM EDTA and 10 mM NaCl, at 4°C for 3

hours with gentle shaking. 0.75 nmol of cold 601 255 bp nucleosomes were added in the elution buffer to maintain the stability of eluted nucleosomes. Eluted nucleosomes were filtered through glass fibre filter under low speed centrifugation (200g) to remove acrylamide particles, washed and concentrated using 100 kDa cutoff spin filters. Eluted nucleosomes, divided into equal aliquots, were further subjected to next round of sliding reaction in the standard remodeling conditions, as described above, for 45 mintutes with increasing amount of RSC (in two fold increments) with the maximum being 15 fmol.

II.5.6 In Gel One Pot assay

The remodeling reaction was performed in a five times scaled up reaction with nucleosomes reconstituted on equimolar mixture of the eight 601.2 mutants. 0.75 pmol (Control reactions with no ATP) or 1.50 pmol (Remodeling reactions) of nucleosomes were incubated with the amount of RSC (35 fmol for control and 70 fmol for remodeling reaction respectively) sufficient to mobilize 45-60% of the nucleosomes. Reactions were stopped by adding 0.05 units of apyrase. Prior to loading on 5% native polyacrylamide gel, 6.25 pmol of cold 255 bp 601.1 middle positioned nucleosomes were added to each reaction as a carrier in order to maintain stability during subsequent procedures. Both control and remodeling reaction were equally divided in five aliquots and resolved on 5% native polyacrylamide gel. Bands corresponding to control unremodeled and unmobilized remodeled nucleosomes were excised, collected in siliconized eppendorf tubes, crushed very gently and immersed with 50 μl restriction buffer (10 mM Tris pH7.6, 10 mM MgCl$_2$, 50 mM NaCl, 1 mM DTT and 100 μg/ml BSA) containing increasing amount of *Hae*III (0.03, 0.12, 0.50, 2.0, 8 units/μl) for 5 minutes at 29°C. The reaction was stopped by addition of an equal volume (50 μl) of stop buffer containing 0.2% SDS and 40 mM EDTA. DNA was eluted from the gel slices, purified as described above, and run on 8% denaturing gel. The quantification of extent of accessibility at different superhelical locations in the nucleosome was performed using Multi-Gauge Software (Fuji).

II.5.7 Gel elution of nucleosomes for AFM analysis

600 fmol of the 255 bp 601.1 nucleosomes were incubated with increasing amount of RSC (30 and 60 fmol respectively) in the remodeling reaction as described above for 30 minutes.

However, the final reaction volumes in this experiment were adjusted to 10 μl to be convenient for loading the samples on gel. After stopping the reaction with apyrase, reaction products were resolved on 5% native polyacrylamide gel. To ascertain the migration of unmobilized and mobilized species, a replicate of the experimental set containing [32]P- labeled 601-255 bp nucleosomes was done and run on the same gel. Nuclesomes were eluted from excised bands, corresponding to control, remodeled and slided species, as described before. Eluted nucleosomes were filtered through glass fibre filter, prior to sample preparation for AFM analysis.

II.5.8 Atomic Force Microscopy, Image Analysis and construction of the 2D maps Lc/ΔL

For the AFM imaging, the nucleosomes were immobilized onto APTES-mica surfaces as described previously (Montel et al., 2007). To automatically analyze AFM images, we have written a Matlab © (The Mathworks, Natick, MA) script based on morphological tools. Using this script we are able to isolate single mono-nucleosomes from other objects present on the image (surface roughness, naked DNA, two connected nucleosomes).

In order to remove the piezoelectric scanner thermal drift, flatten of the image is performed. The use of a height criteria (h>0.5nm where h is the height of the object) allows to avoid the shadow artifact induced by high objects on the image. Then we select nucleosomes based on area criteria and height thresholding. Using a hysteresis height thresholding, we verify the presence of an NCP on each selected objects. For each mono-nucleosome, the NCP center of mass is localized and an Euclidian distance map can be calculated from this origin. After exclusion of the NCP part, the skeletons of the free arm regions are obtained by thinning. By applying the previous distance map, the length of each arm is measured from the NCP centroid. The longest arm is named L_+ and the shortest L_-. DNA complexed length is deduced by $L_c = L_{tot} - L_- - L_+$ where L_{tot} is 255 bp in this case. The position of the nucleosome relatively to the DNA template center is calculated as $\Delta L = (L_+ - L_-)/2$. It is important to notice that the position defined in this way corresponds to the location of the most deeply buried base pair, which might differ from dyad axis position (strictly defined for symmetric nucleosomes).

As the length of each nucleosome arm (L_+ and L_-) is measured from the centroid of the NCP, it is necessary to subtract the crystallographic radius (5.5 nm) of the NCP to get the actual arm length.

To construct the 2D-histogram a 10 bp-sliding box is used. For each coordinates (ΔL_0, L_{c0}) in (0, 150 bp)×(0, 300 bp), nucleosomes with a DNA complexed length included in the range (L_{c0} – 5 bp, L_{c0} + 5 bp) and a position included in the range (ΔL_0 – 5bp, ΔL_0 + 5 bp) are counted. After normalization a smooth distribution is obtained that represents mathematically the convolution of the real experimental 2D-distribution with an 8 bp square rectangular pulse.

During the AFM mobilization assays, we have observed nucleosomes where only one DNA arm is visible. The single DNA arm exhibits the same length as one arm of the over-complexed two-arm nucleosome, and is also clearly different from the slided end-positioned one arm nucleosome. Cryo-EM experiments do not show any of such over-complexed one-arm nucleosome. This type of objects most probably results from the interaction with the functionalized mica surface during the deposition process that might perturb the more labile structure of the 'remosomes'. This type of 'false one arm' nucleosome is very rarely observed on control nucleosomes (-RSC). Accordingly, those objects were discarded during the analysis.

II.5.9 Electron-Cryo microscopy

Samples for electron cryo-microscopy were prepared as described previously (Dubochet et al., 1988). The electron microscopy grids covered with perforated support film were used. The film surface was treated by subsequent evaporation of carbon and carbon-platinum layers and the plastic support was dissolved prior to use. 3 µl of solution was deposited on the grid held in the tweezers mounted in the plunger. The majority of the liquid was blotted away with Whatman No 4 blotting paper and the grid immediately plunged into liquid ethane held at -183°C. The specimen was transferred without re-warming into the electron microscope using Gatan 626 cryo-transfer holder. Images were acquired at 80 kV accelerating voltage either on Philips CM200 using Kodak SO 163 negative films, 66000x direct magnification and 1.5 µm underfocus or Philips Tecnai G2 Sphera microscope equipped with Ultrascan 1000 CCD camera (Gatan) using 14500x direct microscope magnification (0.7 nm final pixel size) and

2.5 μm underfocus. Negatives were developed for 12 minutes in full strength Kodak D19 developer.

II.6 Acknowledgements

II.7 Supplementary Figures
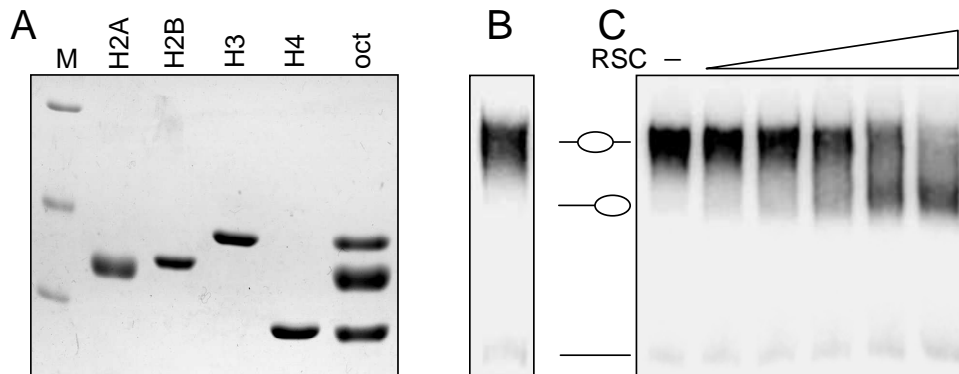
Figure II.S1. The reconstituted nucleosomes are efficiently mobilized by RSC. (**A**) 18% SDS-PAGE of the recombinant histones used for reconstitution and the histone composition (oct) of the reconstituted particles. (**B**) Band shift assay of the reconstituted nucleosomes. Nucleosomes were reconstituted on a 255 bp 601 positioning sequence. Note that under the conditions of reconstitution no free DNA was observed. (**C**) RSC mobilization assay. Reconstituted nucleosomes were incubated with increasing amounts of RSC in the presence of ATP.
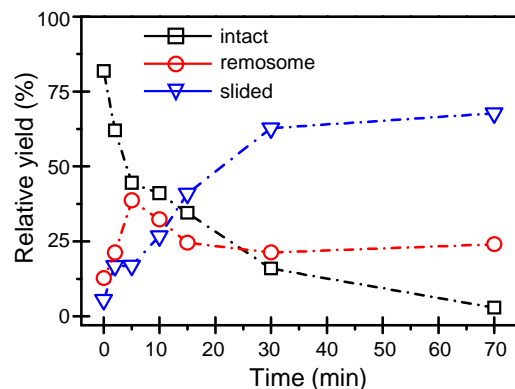
Figure II.S2. AFM experiments show that the remosomes are intermediary particles generated during the RSC nucleosome mobilization reaction. Centrally positioned 601 nucleosomes were incubated with RSC in the presence of ATP and the reaction was stopped at the times indicated. Then the different species present in the reaction mixture were visualized by AFM. The amount of each individual type of particles was measured and after normalization, the percentage of intact nucleosomes, remosomes and slided nucleosomes was presented as a function of the time of the remodeling reaction. Note that the initial increase of remosome amount is followed by a gradual decrease of the amount of this type of particles as the remodeling reaction proceeds.

Chapter III: *Manuscript under preparation.*

Title: Identification and Characterization of Novel Intermediates of SWI/SNF Induced Nucleosome Sliding.

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III.1 Introduction

Chromatin exhibits a repeating structure and its repeating unit, the nucleosome, is a complex of an octamer of the core histones (two each of H2A, H2B, H3 and H4) and ~150 bp of DNA, which is wrapped around the histone octamer in ~1,65 left-handed turns (van Holde, 1988). The structure of both the histone octamer (Arents et al., 1991) and the nucleosome (Luger et al., 1997) was solved by X-ray crystallography. The individual histones consist of a "histone-fold" structured domain and non-structural, highly flexible NH_2-termini, which are protruding from the nucleosome. The nucleosomes are connected by the linker DNA and a fifth histone, the linker histone, is associated with this DNA (van Holde, 1988). The nucleosomal arrays are further folded into the thick 30 nm chromatin fiber and this folding is assisted by the linker histones and the NH_2-core histone termini (Thoma et al., 1979; Wolffe et al., 1997; Hayes and Haysen, 2001). The NH_2-core histone termini are also involved in the assembly of the mitotic chromosomes (de la Barre et al., 2001).

The nucleosomes are stable particles and they interfere with the cellular processes requiring access to genomic DNA (reviewed in Beato and Eisfeld, 1997). The cell uses three main strategies to overcome the nucleosomal barrier and to get access to nucleosomal DNA, namely histone modifications (reviewed in Strahl and Allis, 2000), histone variants (reviewed in Boulard et al., 2007) and chromatin remodeling complexes (reviewed in Becker and Horz, 2002).

Chromatin remodeling complexes are multiprotein assemblies comprising variable number of subunits [Becker and Horz, 2002; Peterson, 2000; Langst and Becker, 2001; Havas et al., 2001). Each remodeling complex contains an ATPase, which possesses a DNA translocase property and is essential for the function of the complex. According to the type of ATPase, the chromatin remodeling complexes are divided in al least four distinct families: SWI2/SNF2, ISWI, CHD and INO80 families (Bao and Shen, 2007; Gangaraju and Bartholomew, 2007). The complexes from the different groups exhibit a common property, they are able to mobilize the histone octamer at the expense of the energy freed by the hydrolysis of ATP. In addition, the complexes from the SWI2/SNF family (SWI/SNF and RSC) induce strong perturbation in the histone-DNA interactions and can evict the histone octamer from nucleosomal DNA (Côté et al., 1998; Lorch et al., 1999). On the other hand,

alterations in the nucleosome structure, induced by the incorporation of some histone variants, affect the capacity of chromatin remodelers to mobilize the histone variant nucleosomes (Angelov et al., 2004; Doyen et al., 2006a; Gautier et al., 2004).

SWI/SNF was the first discovered chromatin remodeling complex (Peterson and Herskowitz, 1992). SWI/SNF in involved several processes, including transcription (Peterson and Herskowitz, 1992), DNA repair (Chai et al., 2005), splicing (Batsche et al., 2006) and telomeric and ribosomal DNA silencing (Dror and Winston, 2004). It consists of ~11 subunits and exhibits a central cavity. The dimensions of the cavity (~15 nm in diameter and ~5 nm in depth) fit well with these of the nucleosome, suggesting that the cavity would be viewed as a nucleosome-binding pocket (Smith et al., 2003). This indicates that SWI/SNF would interact and remodel only one nucleosome at the time.

Despite numerous studies, the mechanism of action of the remodeling complexes is far from being clear. Two different general classes of models were proposed (recently reviewed in (Gangaraju and Bartholomew, 2007). According to the first class of models, DNA moves on the surface of the histone octamer in 1 bp waves. This model is, however, inconsistent with several recent reports (see for review Gangaraju and Bartholomew, 2007). According to the second class of models, favored in the literature, the remodeler creates a bulge on the nucleosomal surface, which is further directionally propagated (Gangaraju and Bartholomew, 2007). Since the dimensions of SWI/SNF are quite large and its contacts with DNA are extensive (the nucleosome is supposed to "fill" the SWI/SNF cavity), a large fragment of DNA could be involved in the SWI/SNF induced bulge formation and indeed, according to the single-molecule experiments the average size of the bulge was found to be about 110 bp (Zhang et al., 2006). Note that each one of the models described the mobilization of the nucleosome as a continuing, non-interrupted process, which is achieved without dissociation of the remodeler from the nucleosome.

In this manuscript we have studied the SWI/SNF nucleosome mobilization mechanism by using a combination of high resolution microscopy techniques (Atomic Force Microscopy (AFM) and Electron Cryo-Microscopy (EC-M)) and novel biochemistry approaches, which allowed measurements with high precision of the DNA accessibility towards restriction enzymes at 10 bp resolution all along the nucleosomal DNA length. We showed that SWI/SNF uses a two-step mechanism to mobilize the nucleosome. The first step involves

pumping towards the center of 15-20 bp DNA from each individual linker, which is accompanied with extensive perturbation in the histone-DNA interactions. This results in the formation of a multitude of nucleosome-like particles, termed remosomes, which contain 175-180 bp DNA associated with the histones. During the second step, the SWI/SNF acts as a true translocase by pumping and releasing DNA in one direction

III.2 Results

III.2.1 The initial step of SWI/SNF nucleosome mobilization mechanism is the perturbation of the histone-DNA interactions and the generation of a non-mobilized nucleosome-like particle associated with ~180 bp of DNA.

By using AFM it was recently shown that during the SWI/SNF nucleosome remodeling reaction, in addition to both the initial non-slided nucleosomes (associated with ~150 bp of DNA) and the completely slided nucleosomes, a third group of particles was observed, which consisted of non-mobilized nucleosome-like particles, but associated with ~175-180 bp of DNA (Montel et al., 2007). The presence of the additional 30-35 bp associated with the histone octamer suggests that the histone-DNA interactions within these non-mobilized nucleosome-like particles might be perturbed. To test this, we used DNase I footprinting. Briefly, we reconstituted centrally positioned nucleosomes by using highly purified recombinant histones and 255 bp 601.1 DNA. Under the conditions used the efficiency of reconstitution was very high (essentially no free DNA was observed in the reconstituted samples) and the reconstituted particles exhibited the typical nucleosomal organization. The centrally positioned ^{32}P-end labeled nucleosomes were incubated with different amounts of SWI/SNF at 29°C with in the presence of ATP, the reaction was arrested with apyrase and run on a 5% native PAGE (Figure III.1).

SWI/SNF units 0 .07 .15 .3 .6 1.2 2.4
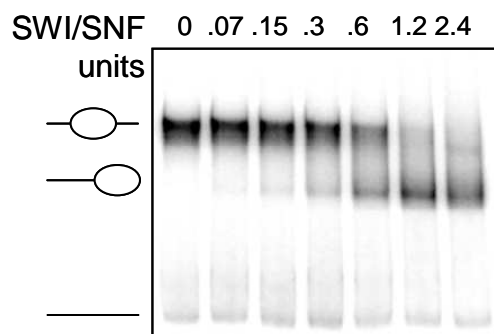
Figure III.1. **Nucleosome mobilization with SWI/SNF.** Centrally positioned nucleosomes on 601.1 DNA were incubated in presence of increasing amount of SWI/SNF (as indicated) for 45 minutes at 29° C. Reactions were arrested by addition of 0.01 units of apyrase and the reaction products were resolved on 5% native PAGE. Positions of unremodeled and slided nucleosomes as well as free DNA are indicated.

Conditions were found where ~50% of the nucleosomes were slided. Then the nucleosomes were incubated with SWI/SNF under these conditions and after arresting the reaction they were treated with increasing amount of DNase I (Figure III.2A). The digested particles were separated on the gel and the upper band (containing the non-slided particles) and the lower band (consisting of slided particles) were cut, the DNA was extracted from the gel slices and run on a 8% denaturing PAGE (Figure III.2B). The digestion pattern of both the slided particles and the non-slided ones, in contrast to that of the control particles (incubated with SWI/SNF in the absence of ATP and gel-eluted after native PAGE), were similar and close to that of naked DNA (Figure III.2B, compare lanes 4-6 and lanes 7-9 with lane 10). Note that this effect was stronger for the slided particles (compare lanes 4 and 5 with lanes 7 and 8). This suggests that the histone-DNA interactions in the non-slided particles are perturbed, which in turn suggests, that the non-slided nucleosome band might consist either of only SWI/SNF remodeled nucleosomes or represent of a mixed population of structurally non-modified particles and SWI/SNF remodeled particles.
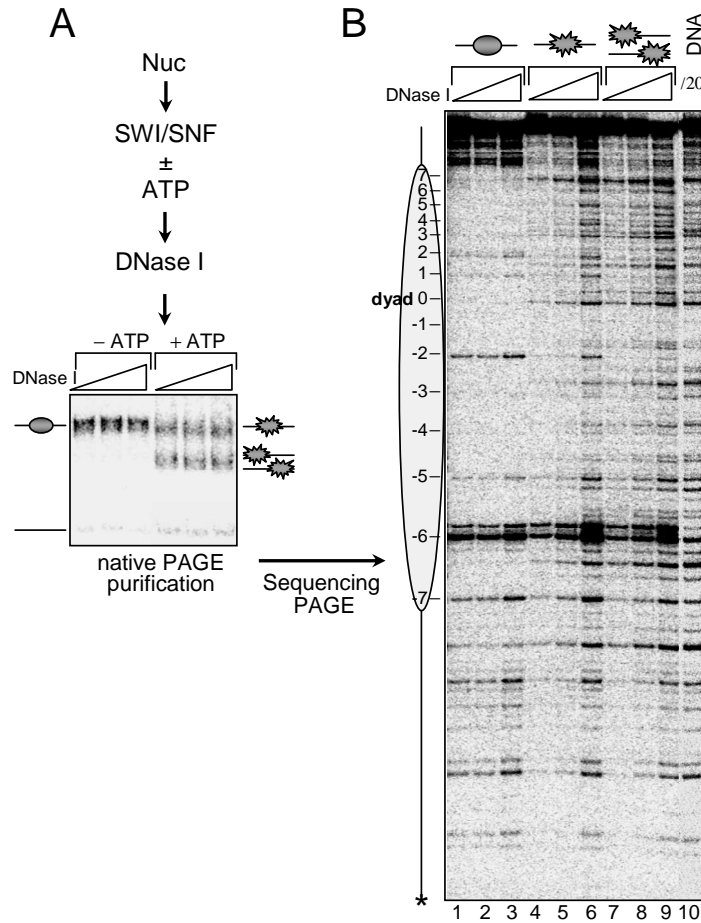
Figure III.2.   **DNase I footprinting analysis shows that nucleosome treatment with SWI/SNF resulted in perturbation of the histone-DNA interactions prior to nucleosome mobilization.** Centrally positioned nucleosomes were reconstituted on 255 bp 601.2 DNA sequence and incubated with SWI/SNF at 29°C in the presence of ATP. Then the reaction was arrested with apyrase and aliquots were incubated with increasing amounts of DNase I for 2.5 minutes at room temperature. After arresting the DNase I digestion reaction, the samples were separated on a 5% PAGE under native conditions. The bands corresponding to either the non-slided particles (upper band) or slided particles (lower band) were excised from the gel, the DNase I digested DNA was eluted from the gel slices and run on a 8% sequencing gel. (**A**) Schematics of the experiment and 5% native PAGE fractionation of SWI/SNF treated and DNase I digested nucleosomes. (**B**) DNase I digestion pattern of control nucleosomes (lanes 1-3) and SWI/SNF treated nucleosomes isolated from the upper band (non-slided particles, lanes 4-6) and the lower band (slided particles, lanes 7-9). On the left side both the position of the histone octamer relative to the ends of the 601 DNA sequence and the nucleosome dyad are indicated. Lane 10, DNase I digestion pattern of naked DNA.

According to previously reported AFM data the remodeled particles would be associated with 175-180 bp of DNA (Montel et al., 2007). We tested this hypothesis by AFM visualization of the SWI/SNF treated nucleosomes isolated from upper and lower electrophoretic bands (Figure III.3). We found that the upper bands contained indeed two types of particles (Figure III.3B row 2). The first types were particles undistinguishable from the control particles (α)

120

with the same free DNA arms in length. The second type (β) exhibited, however, shorter arms but appeared to be still localized close to the center of the DNA fragment (Figure III.3B, compare rows 1 and 2). The nucleosome fraction isolated from the lower electrophoretic band contained only slided (γ) nucleosomes (Figure III.3B row 3).

To precisely measure both the length of DNA associated with the histone octamer and the position of the histone octamer relative to the center of the DNA of one and the same nucleosome, we analyzed several thousands of AFM visualized gel-isolated particles by a specially developed image analysis program which allows us to precisely measure the length of both DNA arms (see chapter II experimental procedure section for detail). The data were presented as L_C and ΔL distributions respectively, where Lc is the length of DNA complexed with the histone octamer and ΔL is the position of the nucleosome relative to the center of the of DNA fragment (Figure III.3 C and D). In these AFM studies, the length of the 601 used for reconstitution was 255 bp and the histone octamer was centrally positioned relative to the ends of DNA, leaving (according to the biochemical characterization) a longer free DNA arm (L_+=56 bp) and a shorter one L_-= 52 bp. For the L_C distribution, Lc was calculated as Lc=L_t – L_+ - L_-, where L_t is the total length of the 601 DNA used for reconstitution whereas; ΔL was calculated as, ΔL= (L_+ - L_-)/2. As seen, the control nucleosomes (α) in the Lc distribution exhibited peak value of Lc= ~150 bp, a result in good agreement with the previous biochemical and AFM data (Montel et al., 2007, Doyen et al., 2006b) as well as with the crystallographic value (Luger et al., 1997). The slided nucleosomes isolated from lower electrophoretic band (γ) also exhibit average DNA complexed length similar to unremodeled control nucleosomes only with a narrower distribution along the L_c axis. This is probably indicative of less fluctuation of one linker DNA arm as compared to nucleosomes with two DNA arms. However, the nucleosomes isolated from SWI/SNF remodeled upper electrophoretic band show an increase in DNA complexed length with the mean value ~165 bp (Figure III.3 C). Considering that the nucleosomes isolated from this band contain an approximately equal mixture of unremodeled as well as remodeled nucleosomes, the mean values of DNA complex length should fall between 150 and 180 bp, hence are in agreement with previously reported values (~180 bp) for SWI/SNF remodeled nucleosomes (Montel et al., 2007). Importantly, the nucleosomes isolated from upper bands (unremodeled as well as remodeled) exhibited the same ΔL distribution profile, confirming that both of these particles were not mobilized (Figure III.3 D). As expected the nucleosome eluted from the lower band

showed a shift in ΔL distribution with the peak value at ~ 50 bp indicative of octamer movement to the end of the DNA (Figure III.3 D).

We conclude that prior to mobilization, SWI/SNF generates particles associated with additional ~30 bp DNA and this results in strong perturbations of the histone DNA-interactions. For simplicity we will refer to these particles, further in the text, as remosomes (remodeled nucleosomes). The remosomes were stable since we have observed them after gel elution and gel eluted remosomes exhibited the same morphology as the remosomes observed directly in the reaction mixture without gel purification (Montel et al., 2007).
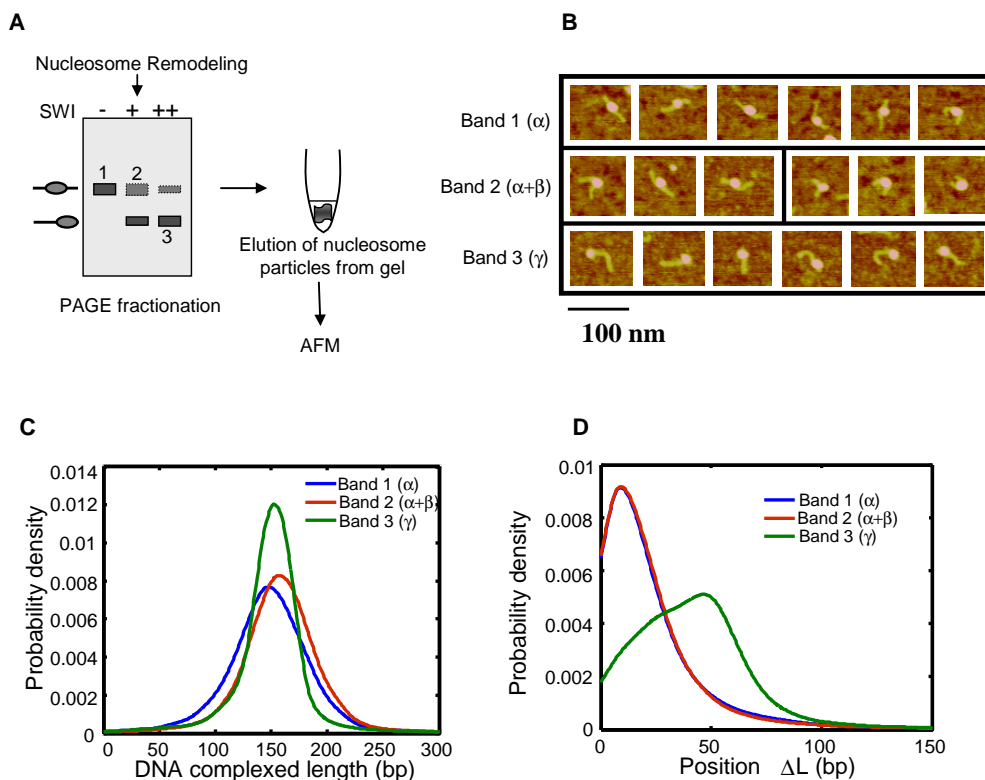
Figure III.3. **SWI/SNF generates non-mobilized nucleosomes particles associated with ~180 bp of DNA.** (**A**) Schematics of the experiment. Centrally positioned nucleosomes were reconstituted on 255 bp 601 DNA sequence. The histone octamer is localized close to the center of the fragment, leaving two free DNA arms with legths of 56 bp (L_+) and 52 bp (L-), respectively. The nucleosomes were incubated with increasing amounts of SWI/SNF for 45 minutes at 29°C and after arresting the reaction with apyrase, they were run on a 5% PAGE. The bands corresponding to either the non-slided (upper band) or slided (lower band) nucleosomes were cut, the nucleosome particles were eluted from the gel slices and analyzed with AFM. (**B**) AFM visualization of the gel-eluted nucleosomes. First row, gel eluted control nucleosomes (incubated in the absence of SWI/SNF); 2nd row, nucleosomes from upper electrophoretic band incubated with SWI/SNF in the presence of ATP; 3rd row, nucleosomes eluted from the lower gel band. (**C**) L_c distribution of the gel eluted nucleosomes from the non-slided and slided nucleosome fractions, L_c is the length of the DNA associated with the histone octamer [$L_c = L_t - (L_+ - L_-)$]. (**D**) ΔL distribution of gel eluted nucleosomes to measure the position of octamer with respect to DNA arms. For unremodeled (α) (n=5806), remodeled ($\alpha+\beta$) (n=4448) and slided (γ) (n= 6410) nucleosome L_c and ΔL distributions (**C** and **D**) are represented in blue, red and green color respectively.

122

III.2.2 Restriction enzyme cleavage of remosome DNA shows dramatic perturbations of the histone-DNA interactions

As mentioned above, the perturbed DNase I digestion pattern of the remosome pointed to a strong perturbation of the histone-DNA interactions. However, if the generation of the remosomes is associated with some very weak (few bases) oscillation of the histone octamer around its initial precise position (resulting in the formation of a multitude of nucleosomes with very slightly changed translational positions, which cannot be detected by AFM), this would also lead to changes in the DNase I digestion pattern. In other words, the alteration in the DNase I footprinting of the remosome could not be unambiguously attributed only to alterations in the histone-DNA interactions. To demonstrate that the remosomes really exhibited strongly perturbed histone-DNA interactions we have developed an approach, termed "In gel one pot assay" (see Figure III.4A). This approach allows the unambiguous detection of the alterations in the histone-DNA interactions at 10 bp resolution all along the nucleosomal DNA and it is based on the restriction enzyme assay developed originally by Wu and Travers (Wu and Travers, 2004). Briefly, eight mutated ^{32}P-end labeled 255 bp 601.2 sequences were used to reconstitute centrally positioned nucleosomes (Figure III.4). Within each one of these sequences a single *Hae III* restriction site was introduced (designated as dyad 0 (d0) to dyad 7 (d7), where the number refers to the number of helical turns from the dyad). Note that each restriction site exhibits identical rotational position with an outward-facing minor groove (Wu and Travers, 2004). Then the nucleosomes were incubated with an appropriate amount of SWI/SNF (in the presence of ATP) to produce 50-60% of mobilized particles (as judged by gel-shift, see Figure III.4A) and the upper electrophoretic band, containing the remosome fraction was excised and in gel digested with increasing amount of *Hae III*. The digested DNA was purified from the gel and run on an 8% PAGE under denaturing conditions. Similar experiment was performed but with control (incubated with SWI/SNF in the absence of ATP) nucleosomes. The gel was dried, the products bands were visualized by exposure on a PhosphorImager and quantified.
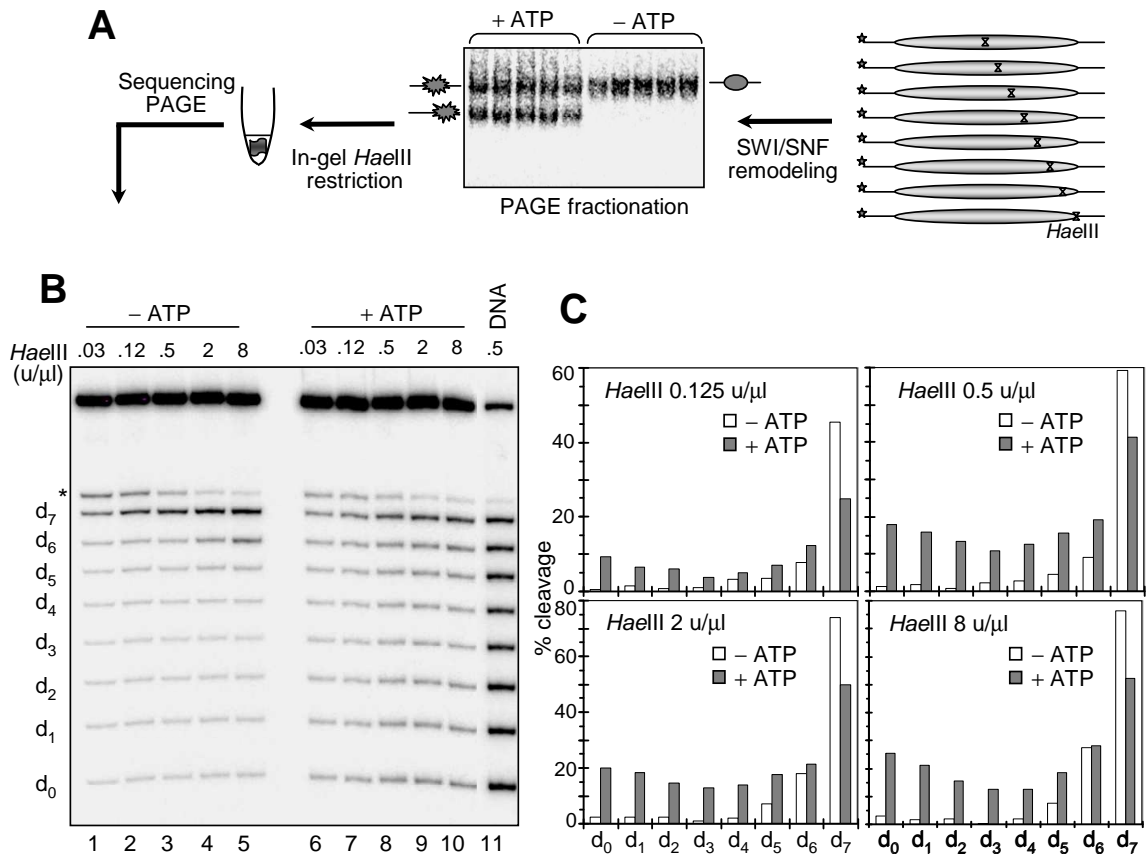
Figure III.4. Measurements of the DNA accessibility to *Hae III* along the nucleosomal DNA length in control and SWI/SNF treated nucleosomes by using the in gel one pot assay. (A) Schematics of the "in gel one pot assay". **(B)** Left panel, *Hae III* restriction nuclease digestion pattern of control nucleosomes (incubated with SWI/SNF in the absence of ATP): right panel, same as (A), but for the treated with SWI/SNF (in the presence of ATP) non-mobilized nucleosomes. After incubation with 2 units of SWI/SNF at 29°C for 45 minutes and separation on a 5% native PAGE , the control and the non-mobilized by SWI/SNF nucleosome fraction were in gel digested with the indicated amount of *HaeIII* for 5 minutes at 29°C. Then the samples were eluted from the gel slices, DNA was isolated and run on 8% PAGE under denaturing conditions. Lane 11, in gel digested naked DNA with 0.5U/μl of *Hae III*. * indicates a fragment which corresponds to an additional *Hae*III site present only in D7 fragment 4 bp away from the d_7 **(C)** Quantification of the data presented in **(B)**.

As seen (Figure III.4 B and C), in the control particles the accessibility to the restriction enzyme strongly decreases from d_7 to d_0. In fact, d_7 and d_6 behaved differently compared to the other dyads since even at the lowest concentration (0.125 U/μl) of *HaeIII*, about 50% of d_7 were accessible to the enzyme and this accessibility increases up to 80% at the highest enzyme concentration (8U/μl). The internally located dyads (from d_4 to d_0) are poorly cleaved at any concentration of *HaeIII* used. These results are in compete agreement with the reported data of Wu and Travers (Wu and Travers, 2004). Upon nucleosome remodeling the *HaeIII* accessibility changed dramatically all along the nucleosome length (Figure III.4 B and C). The accessibility of d_7 is decreased relative to that f the control particles, while that of the

other dyads is strongly increased with highest increase (up to 10-12 folds in the different experiments) observed at d_0. Intriguingly, the *HaeIII* cleavage efficiency distribution showed a parabolic-like shape (Figure III.4C). These data allowed us to conclude that within the remosomes the histone-DNA interactions are markedly perturbed with the strongest SWI/SNF induced perturbations in the vicinity of d_0 close to the center of the particle.

The data from "in gel one pot assay" provided us an average distribution of accessibility across the octamer surface. However, it does not give us kinetics of accessibility at individual superhelical locations. To further elucidate the accessibility profile of remosomes, we gel purified the remosomes and carried out *HaeIII* digestion kinetics experiments in solution with the unremodeled nucleosomes and remosomes (Figure III.5). Note that under our conditions of elution from the gel the remosomes did not disassemble, i.e. ~5% and ~10% of free DNA was observed in the eluted nucleosome and remosome particles solution as judged by both band shift and AFM (data not shown). Under the *HaeIII* digestion condition used (2 units/μl), free DNA was completely digested within 1 minute of digestion (Figure III.5A lane 13). Therefore, these free DNA values, as mentioned above, were subtracted from the calculated % cleavage values for unremodeled nucleosomes and remosomes. The experimental data (Figure III.4B) show that the kinetics curves of the *HaeIII* accessibility of dyads 0-4 for the control nucleosomes are smooth, at the first time point a very small cleavage is detected, which further increases with time with a slow digestion kinetics. The characteristic accessibility profile of unremodeled nucleosomes is preserved, i.e very high accessibility at d_7, a successive drop in accessibility at d_6 and d_5 and very low accessibility observed at d_4-d_0. This indicates that the gel purification does not alter the native nucleosome state after gel purification which is also consistent with our AFM data.
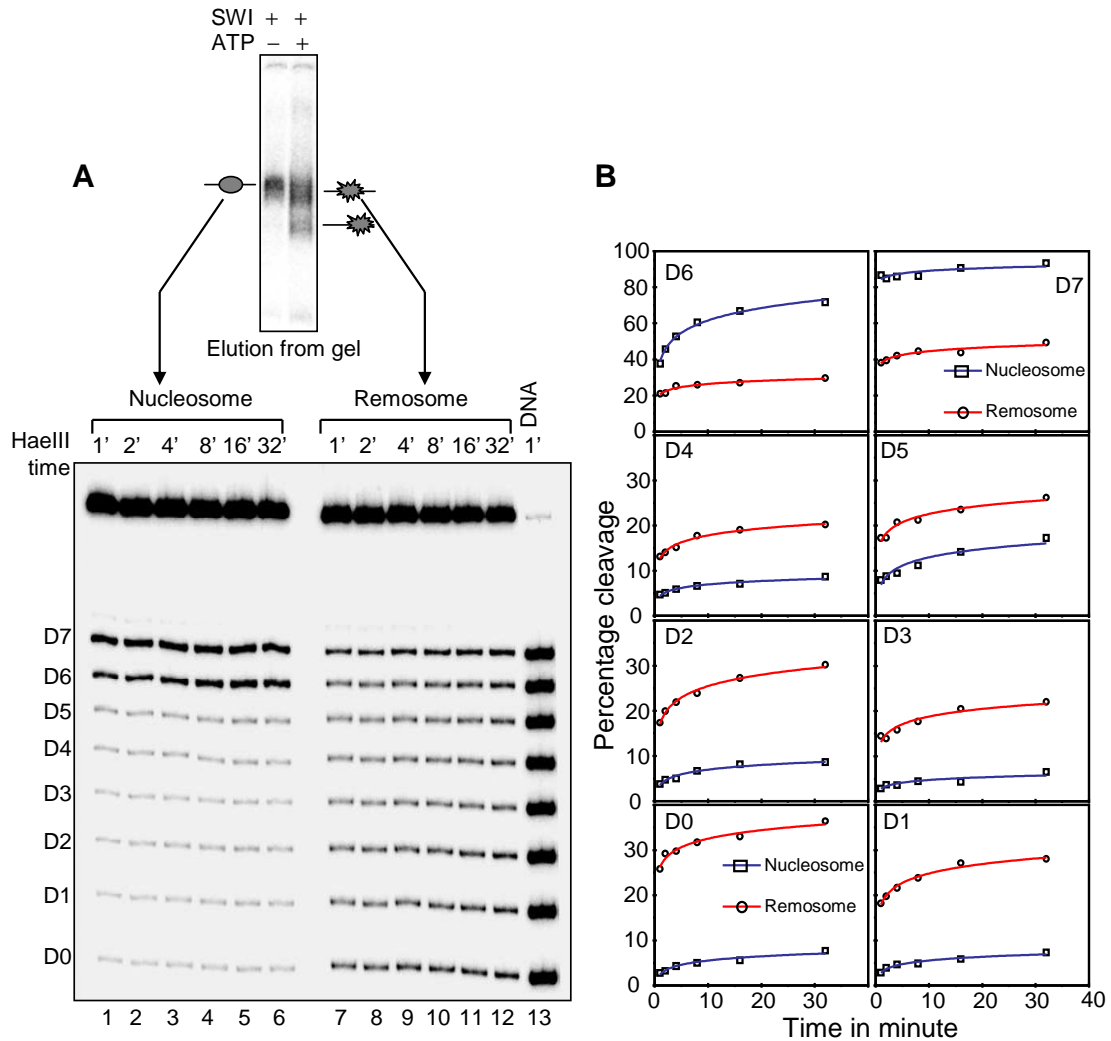
Figure III.5. *Hae III* **digestion kinetics of control nucleosomes and remosomes in solution.** (**A**) Nucleosomes were reconstituted by using the eight ^{32}P-labeled 255 bp 601.2 sequences, each containing a unique *Hae III* site (see figure III.4A) and incubated with 2 units of SWI/SNF for 45 minutes at 29°C. After running of the samples on a 5% PAGE, the control nucleosomes (incubated with SWI/SNF in the absence of ATP) and the SWI/SNF non-mobilized fraction were eluted from the gel in presence of unlabelled 601 nucleosomes. One and the same amount of both types of nucleosomes were digested with 2 U/µl of *Hae III* for different times, DNA from control nucleosomes (left panel lanes 1-6) and remosomes (right panel lanes 7-12) was isolated, purified and run on 8% PAGE under denaturing conditions. The times of digestion and the positions of the different dyads are indicated. Free DNA eluted in presence of same amount of unlabeled 601 nucleosomes was digested for 1 minute (Lane 13). (**B**) Quantification of the data presented in (**A**). Kinetic curves for *HaeIII* accessibility are shown for unremodeled nucleosomes (in blue) and remosomes (in red).

The picture is, however, quite different for the time-dependent *HaeIII* cleavage for the remosome DNA. The kinetics of *HaeIII* digestion of each individual dyad consist of three well defined parts: (i) an immediate cleavage (time point 1 min) indicative of bulge or defect present at that specific location, (ii) a kinetic part (time points 1-8 minutes) indicative of a SWI/SNF induced defect/bulge created in vicinity, leading to transient changes in interaction between the octamer and DNA at this location and (iii) a later part exhibiting relatively slow

126

cleavge comparable of that of control nucleosomes. The percentage of the immediate cleavage varies for different dyads. For all dyads, with the exception of d_7 and d_6, the cleavage is higher than respective one for the control nucleosomes (compare the cleavage of the 1 min time points for control nucleosomes and remosomes for the respective dyads, Figure III.5B). The highest increase in the cleavage is observed in the case of d0, where the cleavage is up to 10-12 folds higher compared to control nucleosomes consistent with the "in gel one pot assay" results.

III.2.3 The remosomes represent a multitude of remodeled nucleosomes, in which each individual particle exhibits a distinctly perturbed path of nucleosomal DNA

The *HaeIII* digestion pattern of the remosomes is strongly suggestive of a multitude of structures, each one exhibiting a distinct altered organization of DNA. To further test this hypothesis, we used Electron Cryo-Microscopy (EC-M) which allowed the visualization of the path of DNA within in an individual nucleosome in unfixed and unstained samples with high resolution (Angelov et al, 2004; Doyen et al, 2006b). Briefly, we incubated centrally positioned nucleosomes with SWI/SNF in the presence of ATP (under conditions where ~30% of nucleosome mobilization is achieved) and then an aliquot of the reaction mixture was vitrified and used for EC-M visualization. The cryo-electron micrographs clearly show three types of structures: (i) centrally positioned nucleosomes, which are undistinguishable from the control ones (Figure III.6A, left panel rows 1, 2 and 3); (ii) completely slided nucleosomes (Figure III.6A left panel, row 4 and 5); note that these nucleosomes are round-shaped and thus, their DNA path appeared to be very similar to this of the control non-slided particles and, (iii) "non-standard" multitude of different structures that we attributed to remosomes. Typically, each such individual structure is larger, shows both shorter free DNA arms and distinct, irregular path of DNA compared to the unremodeled nucleosomes (Figure III.6A right panel, rows 1-5). We conclude that the remosomes are not a single, well defined particle (as the conventional nucleosomes), but instead represent a multitude of structures with distinct and highly perturbed path of DNA. We also studied SWI/SNF mediated remodeling products on trinucleosomes reconstituted on a DNA fragment containing three 601 repeats in tandem (Figure III.6B). Consistent with the data from mononucleosomes, SWI/SNF action on trinucleosomes resulted in generation of typical remosomes like structures characterized by shorter linker DNA and concomitant increase in the diameter of remodeled nucleosomes (Figure III.6B, compare the left panel representing unremodeled

nucleosomes with right panel representing remodeled nucleosomes). Interestingly, within one trinucleosomal template both remodeled as well as unremodeled nucleosomes could be seen. Within the same reaction, a small fraction of trinucleosomes could also be seen in complex with SWI/SNF. Consistent with the dimensions reported in a previous study about SWI/SNF structure (Smith et al., 2003) only one SWI/SNF complex was seen bound to one nucleosome (Figure III.6C). Taken together, this can be taken as evidence that SWI/SNF remodels one nucleosome at a time.

50nm
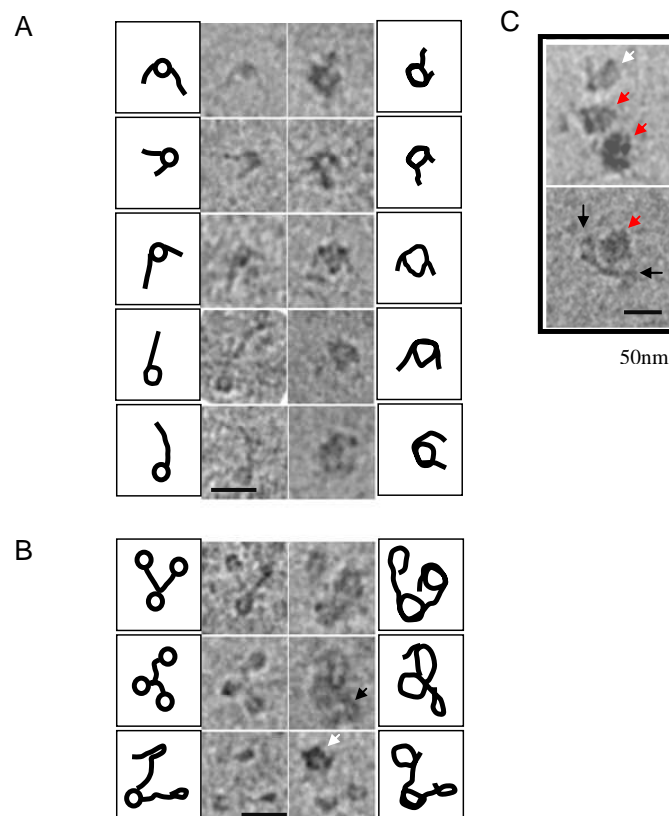
Figure III.6. Observation of different species in SWI/SNF treated mono- and trinucleosomal substrates by Electron Cryo-Microscopy (EC-M). (A) Centrally positioned mononucleosomes were incubated in presence of SWI/SNF and ATP for 30 minutes at 29° C (under these conditions ~40% of nucleosomes were mobilized to the end of the 601 DNA fragment). Left panel shows nucleosomes which are either unperturbed or slided to the end of the DNA fragment by SWI/SNF action. In the right panel nucleosomes with altered structure are represented. **(B)** SWI/SNF is able to alter nucleosomes in a trinucleosomal array. Trinucleosomal template was reconstituted on DNA fragment containing three tandem repeats of 601 sequence. The trinucleosomal array was remodeled in presence of SWI/SNF as in (A). Left panel represents unaltered trinucleosomes while the right panel represents trinucleosomes altered by SWI/SNF. Note that all the nucleosomes are altered by SWI/SNF (right panel row one), only one nucleosome remains unaltered (middle row, indicated by black arrow) or only one nucleosome is altered (bottom row, indicated by white arrow). All the EC-M micrographs are accompanied with line drawing illustrative of the shape of DNA observed in micrographs. (C) SWI/SNF complex associates with a single nucleosome in a trinucleosome array. SWI/SNF bound nucleosomes are indicated by red arrows. Unaltered nucleosomes are indicated by black arrows. An altered but unbound nucleosome is indicated by a white arrow. (Scale bar 50nm)

III.3 Discussion

In this work we have studied the type and structure of the products of the SWI/SNF nucleosome remodeling reaction by using highly resolution microscopy methods combined with novel biochemistry approaches. This has allowed a detailed structural characterization of the SWI/SNF reaction products. In the microscopy study we have used centrally positioned nucleosomes, reconstituted on a 255 bp 601 DNA sequence. These nucleosomes exhibited two free ~50 bp DNA arms, which permitted the visualization of the structural alterations in nucleosomal DNA upon remodeling. We found that, in addition to the mobilized nucleosomes, SWI/SNF generates a multitude of nucleosome-like particles that we called remosomes and which are associated with ~180 bp of DNA, instead of 147 bp of DNA as in the non-remodeled control nucleosomes. Importantly, the AFM data demonstrated that the position of the histone octamer relative to the center of the DNA remained unchanged, indicating that the remosome is generated by SWI/SNF "pumping" of 15-20 bp of DNA from each individual free DNA arm. The "in gel one pot assay" illustrates that the histone-DNA interactions within the remosomes are markedly perturbed all along the remosome DNA. Importantly, the accessibility of dyad 6 and 7 (located at the very end of the nucleosomal DNA) to *HaeIII*, in contrast to those of all the remaining dyads, is decreased in the remosomes, which could be viewed as an evidence for generation of a stronger histone-DNA interactions in the vicinity of this location, i.e. the "pumped" DNA interacting with the histone octamer.

The DNase I footprinting pattern of the remosomes is clearly different from that of the nucleosomes and is similar to free DNA. Since the remosomes appeared to be generated without mobilization of the histone octamer, this points that the remosomes are not a set of well defined particles as the parental nucleosomes are, but instead represent an ensemble of heterogenous structures. The EC-M visualization of the remosomes confirms that this is really the case. A common feature of the remosomes is their larger size than that of nucleosomes. Importantly, each remosome shows an irregular and distinct DNA path, the strongest irregulatities being observed at different locations relative to the center of the particles. This indicates that within each individual remosome, a distinctly localized region with very strongly perturbed histone-DNA interaction should exist. The presence of *HaeIII* immediate cleavage regions all along the remosomal DNA is in perfect agreement with this statement.

Interestingly, under our experimental conditions very few SWI/SNF-nucleosome complexes were detected by both gel shift assays and EC-M, indicating that once the remosome is generated, SWI/SNF dissociates from it.

Taken together, all the above data suggest a two-step mechanism of SWI/SNF nucleosome mobilization. SWI/SNF, as RSC (a complex belonging to the same remodeler's family) exhibits a central cavity (Smith et al., 2003; Leschziner et al., 2007; Asturias et al., 2002). The dimensions of the cavity fit well to these of the nucleosome, suggesting that the nucleosome is localized in the cavity (Smith et al., 2003). We hypothesize that the entry/exit nucleosomal DNA ends and thus, the center of the nucleosome, are oriented towards the solution. Upon hydrolysis of ATP, SWI/SNF generates a bulge in vicinity to the nucleosome center and in this way it perturbs the strongest histone-DNA interactions within the nucleosome. This bulge is generated through "pumping" of DNA from both free DNA arms without repositioning of the histone octamer. The pumped DNA interacts with the histone octamer and a topologically "closed" structure is formed. Once the remosome is generated, SWI/SNF dissociates from it. The bulge is, however, unstable and it can "travel" along the remosomal DNA, but it cannot dissipate since the ends (the "pumped" DNA) of the remosome are "stuck" to the histone octamer surface. In this way a multitude of structures, containing bulges at different sites along the particle are created, and this determines the irregular and distinct DNA shape of each individual remosome.

During the second step of the reaction, SWI/SNF binds again to the remosome, but this time it acts as a true translocase by pulling and releasing DNA around the surface of the histone octamer. Once the histone octamer is moved to the end of the DNA fragment, the excess of remosomal DNA is pulled out and a regular round shaped structure associated with ~150 bp DNA is then formed.

According to the model, the remosome, and not the nucleosome, is used to translocate DNA. This is a crucial feature of the model, since within the remosome the histone-DNA interactions are highly perturbed and thus, the translocation of DNA could be achieved at the expense of less energy. The model also suggests that SWI/SNF would be highly processive and it would not dissociate from the remosome until the histone octamer is moved to the end of the DNA. In addition, the suggested DNA translocation mechanism requires a high flexibility of SWI/SNF in order to be able to bind to the multitude of different remosomes and

translocate DNA. Since the remosomes are larger and very distinct in shape, the translocation step might not be realized through the binding of the remosome to the SWI/SNF central cavity, i.e. the binding of the nucleosome to the central cavity would be required only for the generation of the remosomes.

Earlier reports have suggested that SWI2/SNF2 family of remodelers may generate structurally altered nucleosomes (Fan et al., 2003; Fan et al., 2004; Narlikar et al., 2002). The evidences presented in these reports, could be viewed, however, only as indicative since the data did not allow the differentiation between mobilized and remodeled non-slided nucleosomes. In the present work we have firmly identified, isolated and characterized the remosomes, a population of non-mobilized remodeled nucleosomes with unexpected properties. We predict that the generation of remosomes could be the main *in vivo* function of SWI/SNF as well as of other remodelers of this family. The remosome is relatively stable and could be isolated after separation of the SWI/SNF reaction products on a native gel. In addition, its generation requires only some "pumping" of 15-20 bp of both linkers DNA. This would be low cost and would avoid nucleosome collision, a typical problem encountered in the nucleosome mobilization. Moreover, since the histone-DNA interactions within the remosomes are highly altered, the histones could be easier evicted from the remosomes compared to the conventional nucleosomes and the creation of histone-free regions would be facilitated. We predict that the generation of such relatively long-lived nucleosome-like particles within the cell may significantly assist several processes, including DNA repair and transcription.

III.4 Experimental Procedures

III.4.1 Nucleosome remodeling reactions

Typical remodeling reactions were performed with 150 fmol of nucleosomes in remodeling buffer (RB) 10 mM Tris pH 7.4, 5% glycerol, 1 mM rATP, 2.5 mM MgCl$_2$, 1 mM DTT, 100 µg/ml BSA, 50 mM NaCl, 0.01% NP40) in a volume of 7.5 µl at 29° C. For sake of convenience SWI/SNF amounts are expressed in units. The SWI/SNF units were defined as described before (Angelov et al., 2006). However, under the experimental conditions

described nucleosomes were always in 10-15 molar excess with respect to SWI/SNF concentration even under the highest concentration of SWI/SNF used.

III.4.2 Dnase I footprinting assay

The remodeling reaction was performed in Remodeling buffer (RB) 10 mM Tris pH 7.4, 5% glycerol, 1 mM rATP, 2.5 mM $MgCl_2$, 1 mM DTT, 100 µg/ml BSA, 50 mM NaCl, 0.01% NP40) in a volume of 7.5 µl at 29° C for 50 min. The control reactions did not receive ATP. 450 fmol (Control reactions) or 900 fmol (Remodeling reactions) of Nucleosomes reconstituted on ^{32}P- end labelled 255 bp 601.2 DNA were incubated with the amount of SWI/SNF sufficient to mobilize ~50% of the nucleosomes. Reactions were stopped by addition of 0.03 Units of Apyrase and 3µg of plasmid DNA. Reaction products were divided into three equal aliquots and increasing amount of DNaseI (0.6, 0.12, 0.25 for control nucleosomes; 0.12, 0.25 and 0.5 units for remodeled nucleosomes respectively) was added to remodeleled or control nucleosomes. EDTA was added to 20mM to stop the DNaseI cleavage. Unmobilized and mobilized fractions were resolved on Native PAGE (29:1) in 0.25X TBE. Bands, corresponding to Unremodeled, Remodeled-unmobilized and Slided nucleosomes were excised from the gel, DNA was eluted, filtered, deproteinized through phenol:chloroform treatment, precipitated and run on 8% Denaturing PAGE.
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III.4.3 Restriction enzyme assay on gel eluted nucleosome

Centrally positioned 150 fmol of 601.2 nucleosomes were incubated with SWI/SNF in the remodeling reaction as described above. Reaction was stopped 45 minutes by addition of 0.01 units of Apyrase and 1 µg of plasmid DNA, as under these conditions the non-mobilized fraction contains essentially remodeled nucleosome particles. Reaction products were resolved on 5% native polyacrylamide gel. Bands, corresponding to unmobilized fractions (Unremodeled as well as remodeled) were excised. Excised bands were then cut in small pieces and soaked in 80 µl Elution Buffer (EB) containing Tris 10 mM pH7.4, 0.25 mM EDTA and 10 mM NaCl, at 4°C for 3 hours with gentle shaking. 0.75 pmol of cold 601 255 bp nucleosomes were added in the elution buffer to maintain the stability of eluted nucleosomes. Eluted nucleosomes were filtered through glass fibre filter under low speed centrifugation (200g) to remove acrylamide particles, washed and concentrated using 100 kDa

cutoff spin filters. Eluted nucleosomes were adjusted to buffer conditions of the restriction digestion conditions (10 mM Tris pH7.6, 10 mM $MgCl_2$, 50 mM NaCl, 1 mM DTT and 100 µg/ml BSA). *HaeIII* was added to 2 units/µl and the reaction was allowed to proceed at 29° C. At indicated time points aliquots were taken and the reaction was stopped by addition of 0.1% SDS and 20 mM EDTA. DNA was extracted through phenol:chloroform, precipitated and run on 8% denaturing PAGE. Gels were dried, autoradiographed, scanned on phosphorimager and quantified using Multigauge software (Fuji).

III.4.4 Atomic Force Microscopy

For the AFM imaging, the nucleosomes were immobilized onto APTES-mica surfaces as described previously. Image acquisition and analysis were done as described in chapter II. DNA complexed length (L_c) and position (ΔL) distributions were constructed as described (Montel et al., 2007).

Other experimental procedures were essentially similar to and as described in chapter II.

Chapter IV: *Manuscript under preparation*

Title: H2A Docking of H2A is essential for SWI/SNF and RSC induced nucleosome sliding through generation of remosome intermediates.

IV.1 Introduction

Nucleosomes, the fundamental repeating unit of chromatin, consist of an octamer of histones containing two copies of each of H2A, H2B, H3 and H4. Around this histone core about 146 bp of DNA is wound in 1.65 superhelical turns (Luger et al., 1997). The organization of DNA into chromatin is generally repressive for various DNA related transactions like replication, transcription, repair and recombination. Two well understood modes to overcome this nucleosomal barrier are covalent modifications of histones and ATP dependent chromatin remodeling (Strahl and Allis, 2000; Becker and Horz, 2002). An emerging concept in regulation of chromatin dynamics is incorporation of histone variants within the nucleosome (Boulard et al., 2007)

Histone variants are nonallelic isoforms of conventional histones (van Holde, 1988 and Russanova et al., 1989). The primary structure of histone variants shows various degrees of homology with the corresponding conventional histone (Malik and Henikoff, 2003). Incorporation of histone variants within the nucleosome imparts new structural and functional properties influencing vital cellular processes like transcription, repair, cell division and meiosis etc (Suto et al., 2000; Abbott et al., 2001; Angelov et al., 2003; Gautier et al., 2004; Ahmad and Henikoff, 2002; Ausio and Abbott, 2002; Kamakaka and Biggins, 2005; Boulard et al., 2007).

The histone H2A family encompasses the greatest diversity of variants among core histones (Redon et al., 2002; Sarma and Reinberg 2005; Boulard et al., 2007). The members of histone H2A family (H2A.1, H2A.X, H2A.Z, mH2A and H2A.Bbd) exhibit significant sequence variability at both N and C terminal ends (Ausio and Abbott, 2002; Ausio, 2006). While the implications of N terminal heterogeneity still remains unclear, most of the recent work has been focussed on C terminal domain variations. Initially, Eickbush et al., (1988) demonstrated that the carboxy terminal tail of H2A is essential for the stability of nucleosomal particles and that the H2A-H2B dimer displays a significant decrease in the affinity for the $(H3-H4)_2$ tetramer when the terminal 15 amino acids are removed by an endogenous protease.

Interestingly, one of the latest described H2A variant, H2A.Bbd exhibits a similar C-terminal truncation (Chadwick and Willard, 2001).

H2A.Bbd (<u>B</u>arr <u>b</u>ody <u>d</u>eficient) derives its name from its property to be excluded from the female inactive X chromosome. It is found to be localized to histone H4 acetylated regions in the nucleus thus suggesting its association with transcriptionally active euchromatin. It is quite divergent as the primary sequence exhibits only 48% homology to the conventional H2A counterpart (Chadwick and Willard, 2001). Major structural hallmarks of H2A.Bbd as compared to conventional H2A are presence of a stretch of 6 arginine residues at the N terminal, presence of only one lysine residue as compared to 14 lysine residues in H2A, and absence of C terminal tail and the very last segment of the docking domain. Moreover, most of the amino acid variations are concentrated in the docking domain (Chadwick and Willard, 2001; Bao et al., 2004; Doyen et al., 2006b). In the conventional H2A, amino acids 82-119 form a distinct ladle shaped structure (the docking domain) which is involved in organizing last turn of DNA through guiding the H3αN helix. The short α-C helix (amino acids 92-96) of H2A forms a short β sheet interaction with C-terminal region of H4 (amino acids 95-102). The whole docking domain of H2A constitutes about 2000 Å^2 of interaction area with (H3-H4)$_2$ tetramer (Luger et al., 1997).

Not surprisingly, incorporation of H2A.Bbd results in profound changes in structural and functional properties of nucleosomes. These changes include, a more relaxed structure and organisation of only ~130 bp of DNA in contrast to ~147 bp on canonical NCPs suggesting release of ~10 bp nucleosomal DNA from each end of the octamer (Doyen et al., 2006b). Moreover, the H2A.Bbd-H2B dimer is less strongly associated with the tetramer resulting in lower stability of the nucleosomes containing H2A.Bbd (Bao et al., 2004; Doyen et al., 2006b; Gautier et al., 2004).

These structural changes result in increased transcription factor access and a less prohibitive chromatin to transcription (Angelov et al., 2004). Note that these changes are usually associated with action of ATP dependent chromatin remodeling machines. It is intriguing, however, despite of having a relaxed structure the H2A.Bbd containing nucleosomes or chromatin are refractory to action of ATP dependent remodelers like SWI/SNF and ACF. This property was largely attributed to the presence of a defective docking domain in H2A.Bbd (Angelov et al., 2004; Doyen et al., 2006b). However, SWI/SNF action on

H2A.Bbd nucleosomes resulted in a partial increase in restriction enzyme accessibility and base excision repair (Angelov et al., 2004; Menoni et al., 2007).

The aforementioned studies strongly indicate the importance of H2A docking domain and C-terminal region in the process of nucleosome remodeling. In this work, we have tried to further elucidate the role and the mechanistic aspects of involvement of these domains in nucleosome remodeling by SWI/SNF and RSC, two of the best characterized ATP dependent chromatin remodeling complexes from yeast.

IV.2 Results

IV.2.1 Nucleosome reconstitutions with H2A C-terminal deletion, chimeric and variant proteins

In order to understand the role of H2A docking domain and C-terminal part in nucleosome remodeling we first made serial deletion mutants using the *X. laevis* N-terminal HA-tagged H2A protein as the parent clone. A chimeric protein H2A.ddBbd was constructed in which the docking domain of H2A was replaced with the docking domain of H2A.Bbd (H2A.ddBbd). As a control full length H2A and H2A.Bbd were also used. Alignment of human H2A.1 and H2a.Bbd are shown in figure IV.1A. Truncation points in deletion mutants are indicated by arrowheads (in red) above the H2A.1 sequence. All the proteins were bacterially expressed and purified in denaturing conditions as described in *materials and methods* section. The purity of the recombinant proteins was checked by 18% SDS-PAGE (Figure IV.1B). We next checked if the mutant proteins could be reconstituted in nucleosomes. For this, nucleosome reconstitutions were performed using salt dialysis method and replacing conventional H2A by mutant proteins in the reconstitution mixtures containing all the four histones and a *NotI* digested 601 DNA. This DNA fragment strongly positions nucleosomes at one end and is an ideal substrate for DNase I based footprinting assays. All the mutants and variant H2A proteins were efficiently reconstituted in the nucleosomes as shown in figure IV.1C. Under the reconstitution conditions very little free DNA was observed (with the exception of Δ79 nucleosomes where the amount of free DNA was slightly higher). This evidences for good incorporation of mutant histones and reconstitution of *bona fide* nucleosomes. Note that the nucleosomes containing deletion mutants of H2A exhibit a slower migration in the gel and

this tendency increases with successive deletion in the C-terminal region. We attribute this to change in conformation of linker DNA which affects the migration in the gel.
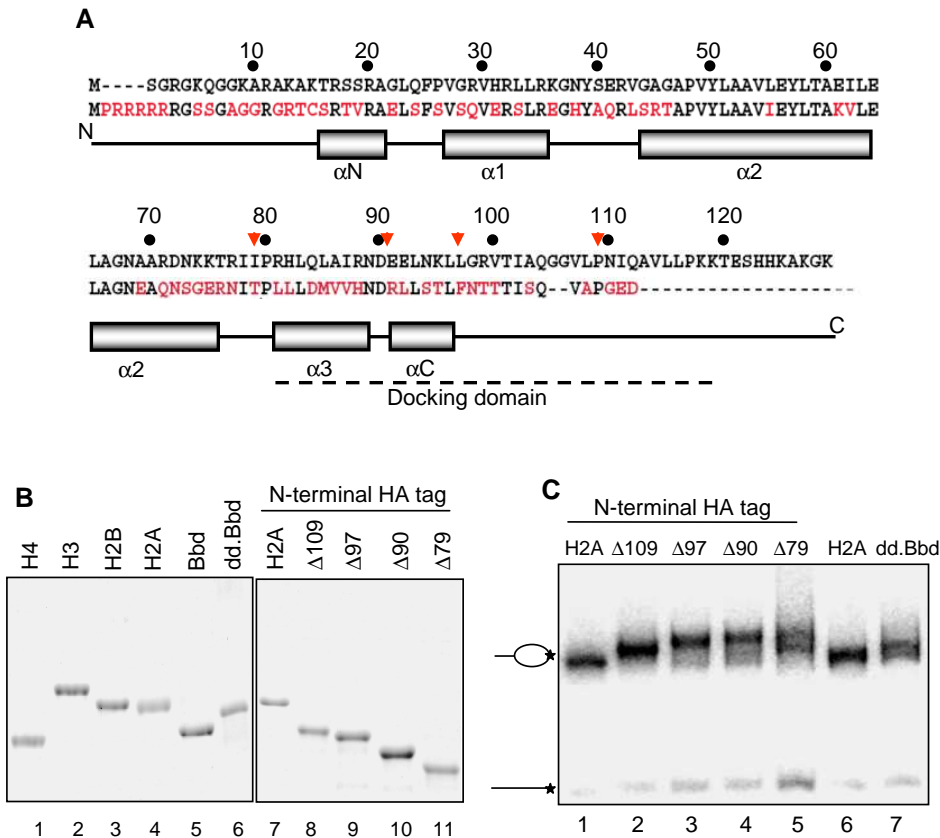
Figure IV.1. Reconstitution of nucleosomes with H2A C terminal deletion and chimeric proteins. (**A**) Alignment of human H2A.1 and H2A.Bbd proteins. Domain structure of histone H2A is represented in the form of cartoon drawing below the sequence. H2A docking domain is represented as dotted line below the sequence. Inverted arrowheads above the H2A sequence (in red) represent the last amino acid in truncated proteins. In H2A.ddBbd chimeric protein the docking domain and the last C-terminal part was replaced with docking domain of H2A.Bbd. (**B**) 18% SDS PAGE of different histones and H2A mutant proteins. All the proteins were bacterially expressed in denaturing condition and purified from inclusion bodies using SP-sepharose medium. Note that the proteins in lane 7-11 are N terminal HA tagged. However, it does not change the properties of nucleosomes (Discussed in text) (**C**) EMSA of the end-positioned conventional (lane 1 and 6), variant (lane 8) and mutant (lanes 2, 3, 4, 5 and 7) nucleosomes, reconstituted on NotI-restricted and 3'-labeled 601 DNA (upper strand) to be used in DNase I footprinting experiments. The 3'-^{32}P label position is indicated by an asterisk. Positions of nucleosomes and free DNA are indicated.

IV.2.2 Changes in C-terminal region of H2A results in structural perturbations in nucleosomes

The migration profile of nucleosomes containing mutant and chimeric H2A (Figure IV.1C) is indicative of structural changes in the nucleosomes they are incorporated in. To test this possibility we performed DNase I footprinting assay (Figure IV.2A). This assay is very useful

in deciphering site specific changes in the conformation of nucleosomal DNA. DNase I digestion of canonical nucleosomes gives a 10 bp repeat, typical for 601 nucleosomes, indicative of minor groove of nucleosomal DNA facing towards the solution (lanes 2-4). Incorporation of H2A Δ109 in the nucleosome showed no major structural perturbations. However, subtle changes were observed in the vicinity of nucleosomal dyad (lanes 5-7). Further deletion of C teminal residues, i.e H2A Δ97 which lacks α-C helix and H2A Δ90 which lacks all of the C-terminal tail as well as the last two α helices, results in clear perturbation in the conformation of nucleosomal DNA (lanes 8-10, 11-13). Prominent changes are indicated by the asterisk. Similar perturbations are also seen when all of H2A C-terminal as well the docking domain is completely deleted (H2A Δ79) as seen in lanes 14-16 or replaced with docking domain of H2A.Bbd (lanes 17-19) leading to a DNase I digestion profile quasi-identical to H2A.Bbd nucleosomes (lanes 20-22). Note that the N-terminal HA tag on deletion proteins does not contribute to these changes as HA tagged conventional H2A and untagged H2A containing nucleosomes exhibit identical DNase I digestion profile (Compare lane 4 to 23).

In parallel, we performed OH° footprinting (Figure IV.2B) on the nucleosomes containing H2A.Bbd (lane 2), H2A.ddBbd (lane 3), and H2A Δ79 (lane 4). A 10 base periodic repeat was found similar to nucleosomes containing canonical H2A (lane 1) confirming the wrapping of DNA around the histone octamer. This is not surprising as either type of nucleosomes may not pose a steric hindrance towards OH° as seen with DNase I (Hayes and Lee, 1997).

An interesting phenomenon observed here is the progressive appearance of specific bands in DNase I profile with progressive deletion of C-terminal region of H2A. As described before the C terminal domain H2A perform two major functions (i) organisation of last turn of DNA through interaction with H3 αN helix and (ii) formation of a β-sheet interaction with C-terminal of H4, thus contributes to the strength of dimer-tetramer interaction (Luger et al., 1997). Note that histone octamer is not stable at physiological salt conditions (Eickbush et al., 1978). This is due to weak nature of interactions between H2A-H2B dimer and (H3-H4)$_2$ tetramer and wrapping of DNA contributes significantly in maintaining the interaction between the two (Luger et al., 1997; Bao et al., 2004). Therefore, the perturbations observed deep inside the nucleosome by DNase I footprinting could be largely attributed to weakened dimer-tetramer interactions. This weakening could be caused indirectly by (i) loss of

organization of last turns of DNA in mutants lacking the last part of C terminal domain and (ii) by directly affecting the strength of dimer-tetramer interface in mutants lacking the base of the docking domain.
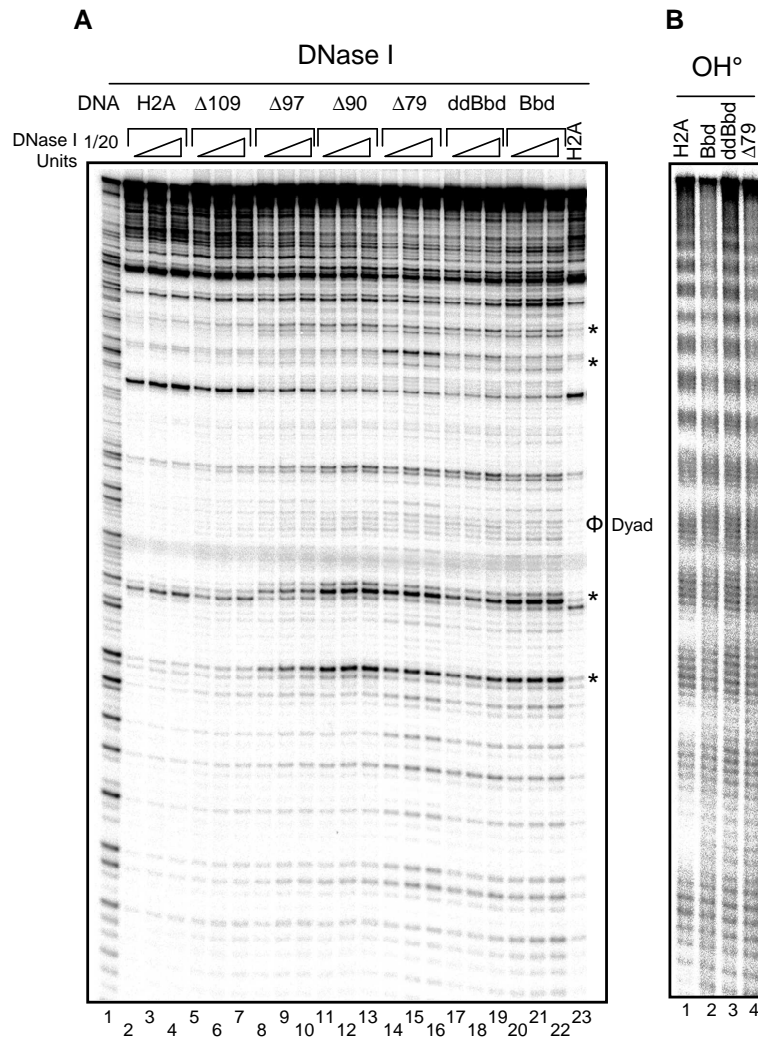
Figure IV.2 Biochemical characterization of conventional, variant and mutant nucleosomes by DNase I and OH° footprinting. Nucleosomes, described in figure IV.1C, were subjected to DNase I or OH° footprinting. After stopping the reaction DNA was deproteinized, ethanol precipitated and run on 8% denaturing PAGE. **(A)** Nucleosomes were digested with increasing amount of DNase I (0.2, 0.3 and 0.45 units) for 2.5 minute at room temperature (lane 2-23). Free DNA (lane1) was digested with 0.01 units of DNase I in the same conditions. As a control of nucleosomes containing HA tagged H2A (lane 2-16), DNase I digested untagged H2A nucleosomes (Lane 23) were also run. Major structural perturbations are indicated by asterisk (*). Position of nucleosomal dyad is indicated by Φ. **(B)** In parallel, conventional H2A (lane 1), H2A.Bbd (lane 2), H2A-dd.Bbd (Lane 3), and H2A-Δ79 (lane 4) nucleosomes were subjected to OH° footprinting.

IV.2.3 The base of H2A docking domain is essential for SWI/SNF mediated mobilization of nucleosomes

It is well documented that H2A.Bbd containing nucleosomes are refractory to SWI/SNF and ACF mediated (Angelov et al., 2004) as well as heat induced mobilization (Bao et al, 2004). Moreover, the observation that truncations in H2A C-terminal domain and swapping of H2A docking domain with that of H2A.Bbd result in perturbations similar to H2A.Bbd containing nucleosomes led us to test if these structural changes result in affecting SWI/SNF catalyzed mobilization of nucleosomes. To this end, we performed a sliding assay with nucleosomes containing truncated H2A proteins or chimeric H2A protein with swapped docking domain. Nucleosomes were reconstituted on a 255 bp 601 DNA. This DNA fragment strongly positions nucleosome in the centre and sliding of nucleosomes to the end of the DNA could be ascertained by faster migration of the slided species in native PAGE. All the nucleosomes were incubated with increasing amount of SWI/SNF for 45 minutes at 29° C. Reactions were stopped by addition of apyrase and the reaction products were resolved on 5% native PAGE (Figure IV.3A). Conventional H2A containing nucleosomes are slided efficiently by SWI/SNF as seen in figure IV.3A. However, nucleosomes containing H2A truncated till the Δ90 are also slided with similar efficiency by SWI/SNF and very little decrease in sliding efficiency was observed. The results were further confirmed by quantitation of the gel pictures in figure IV.3A and the percentage of slided species was plotted against SWI/SNF units (Figure IV.3B). The situation, however, changes drastically when the very last part of the docking domain is deleted (Δ79) or when the H2A docking domain is swapped with H2A.Bbd. No sliding was observed even with highest concentration of SWI/SNF.

We also validated the results of H2A.ddBbd nucleosomes using AFM analysis. Briefly, centrally positioned 601 255bp H2A.ddBbd nucleosomes were remodeled in presence of 2 units of SWI/SNF in conditions similar to the sliding assays and the reaction mixtures were deposited on treated mica surface for AFM imaging in air. Using specially designed software (see chapter II experimental procedure section for detail) we were able to precisely measure both the length of DNA associated with the histone octamer (L_c) and the position of the histone octamer relative to the center of the DNA (ΔL) of one and the same nucleosome. To get statistically significant results we analyzed several hundreds of AFM visualized particles. The data were presented as ΔL and L_C distributions respectively (Figure IV.4 A and B).

141

Similar to the sliding assay conditions, in this AFM experiment, the length of the 601 DNA used for reconstitution was 255 bp and the histone octamer was centrally positioned relative to the ends of DNA, having a longer free DNA arm $L_+=56$ bp and a shorter one $L_-= 52$ bp. For ΔL distribution, ΔL was calculated as $\Delta L= (L_+ - L_-)/2$. For the L_C distribution, Lc was calculated as $Lc=L_t -L_+ - L_-$, where L_t is the total length of the 601 DNA used for reconstitution.

As seen, the control (incubated in absence of ATP) as well as remodeled H2A.ddBbd nucleosomes exhibited a wide ΔL peak distribution (in contrast to conventional nucleosomes, see figure III.3C). Importantly, it does not change significantly by action of SWI/SNF, confirming SWI/SNF is unable to mobilize these particles to the end of the DNA ($\Delta L=60$bp) (Figure IV.4 A), consistent with the results obtained from nucleosome sliding assays. The Lc distribution profile of control nucleosomes shows a peak value at ~130 bp meaning that only 130 bp of DNA is attached to the histone octamer. Similar values were obtained in an AFM study on H2A.Bbd nucleosomes (Montel et al., 2007) further emphasizing the role of docking domain of H2A.Bbd in open structure of these nucleosomes. The remodeled H2A.ddBbd nucleosomes, however, show an increase in DNA complexed length (mean value ~145 bp) indicating towards structural perturbations imparted by SWI/SNF (Figure IV.4B). Note that the L_c distribution of remodeled nucleosomes is very wide. This strongly indicates fluctuations of linker DNA arms and suggests that the action of SWI/SNF on H2A.ddBbd nucleosomes results in pumping of linker DNA inside the nucleosome and that the interaction of the DNA to the octamer remains dynamic.

The data from nucleosome sliding assays and AFM analysis, taken together, proves that the docking domain of H2A is required for nucleosome mobilization mediated through SWI/SNF.
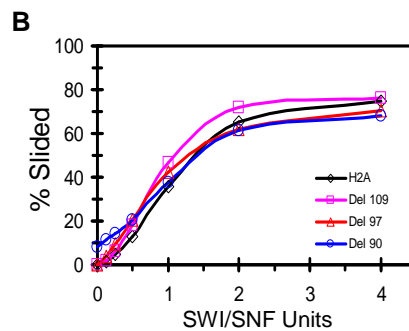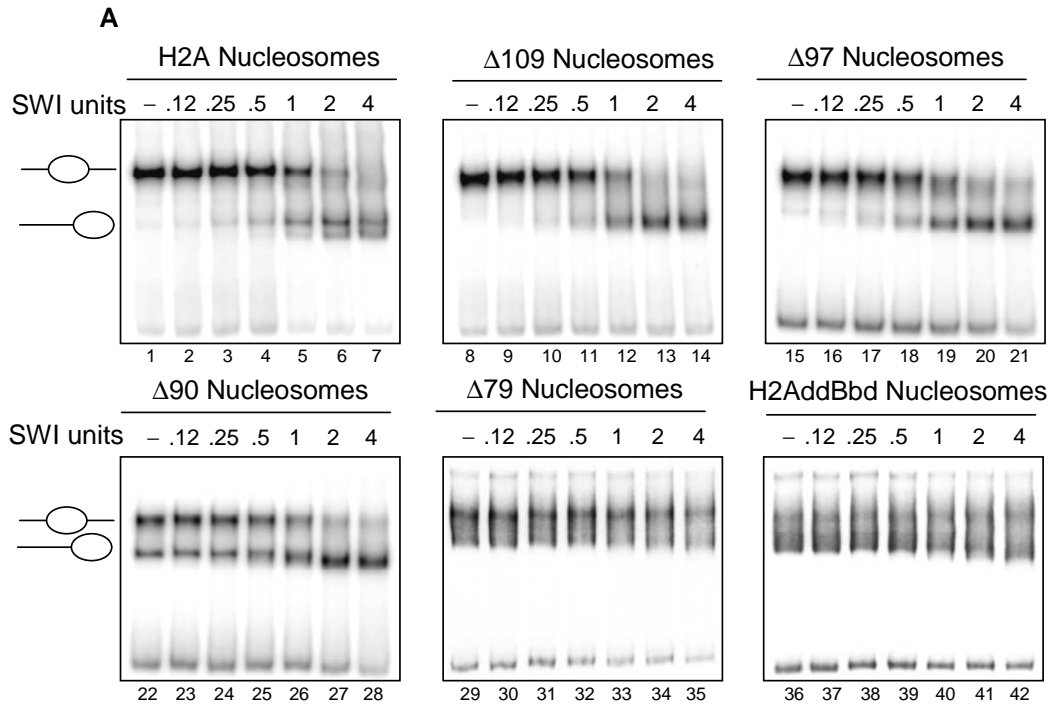
Figure IV.3. H2A docking domain is essential for SWI/SNF induced nucleosome mobilization. **(A)** Centrally positioned conventional H2A (upper row left panel), Δ109 (upper row middle panel), Δ97 (upper row right panel), Δ90 (lower row left panel), Δ79 (lower row middle panel) and H2A.ddBbd (lower row right panel) containing nucleosomes on a 255 bp 601 DNA were incubated with increasing amounts of SWI/SNF in presence of 1mM ATP for 45 minutes at 29° C. Lanes 1, 8, 15, 22, 28 and 36 represent control reactions for respective nucleosomes without added SWI/SNF. Reactions were stopped by addition of 0.01 units of apyrase. Samples were resolved on 5% native PAGE. Gels were dried and visualized by exposure on a PhosphorImager. Positions of unmobilized and slided nucleosomes in the gel are shown by cartoon drawing. **(B)** Quantitation of gel data for conventional H2A, Δ109, Δ97 and Δ90 nucleosomes presented in A.
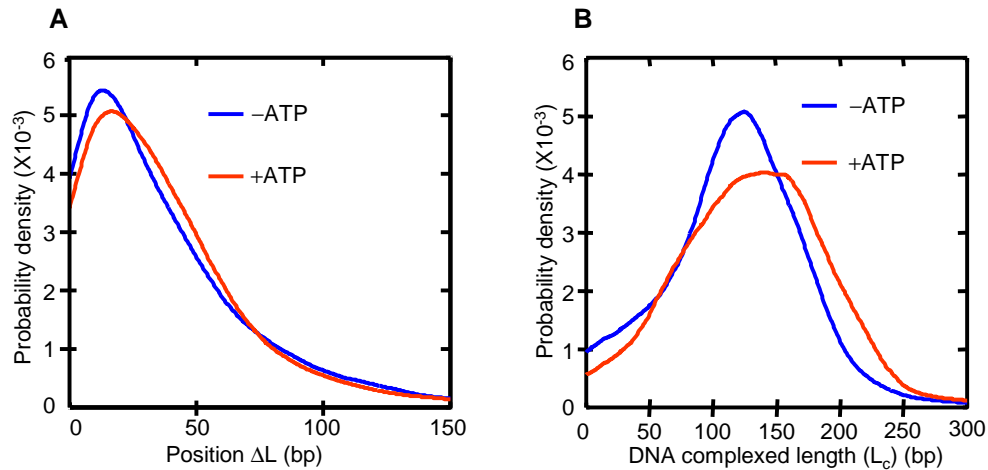
Figure IV.4. **AFM analysis of SWI/SNF induced remodeling on H2A.ddBbd nucleosomes** Centrally positioned nucleosomes were reconstituted on 255 bp 601 DNA sequence. The histone octamer is localized close to the center of the fragment, leaving two free DNA arms with legths of 56 bp (L_+) and 52 bp (L-), respectively. The nucleosomes were incubated with 2 units of SWI/SNF for 45 minutes at 29°C and after reaction products were analyzed by AFM. (**A**) ΔL distribution of control (incubated in absence of ATP) and SWI/SNF remodeled nucleosomes to measure the position of octamer with respect to DNA arms, $\Delta L = (L_+ - L_-)/2$ (**B**) L_c distribution of the unremodeled and remodeled nucleosomes, L_c is the length of the DNA associated with the histone octamer [$L_c = L_t - (L_+ - L_-)$]. For unremodeled nucleosomes N=1510, for remodeled N=585.

IV.2.4 RSC is more sensitive to perturbations in the docking domain of H2A

The yeast RSC (Remodels Structure of Chromatin) complex is another complex from yeast belonging to the SWI2/SNF2 family (Cairns et al., 1996). It is shown to be similar in all the biochemical activities associated with SWI/SNF complex. However, they are not redundant and exhibit different functional properties *in vivo* (Becker and Hörz, 2002). This prompted us to test the effect of H2A C-terminal defect on RSC mediated nucleosome sliding as well. As in previous experiment, different nucleosomes were incubated with RSC in presence of ATP and sliding efficiency was checked by standard gel shift assay (Figure IV.5A). As with SWI/SNF, the nucleosomes containing full length H2A were mobilized efficiently (lanes 2-7). 2.4 units of RSC were sufficient to slide nucleosomes to saturation in 45 minutes. However, contrary to SWI/SNF, C-terminal truncations of H2A exhibit a profound effect on RSC mediated sliding (Figure IV.5A, lanes 16-21 and 23-28) which is clearly represented in conditions when nucleosomes were incubated with 1.2 units of RSC. In this condition, while RSC is able to slide ~60% of canonical nucleosomes, only ~30% and ~15% of Δ97 and Δ90 nucleosomes were slided respectively (Figure IV.5B). Even with highest amount of RSC the reduction in sliding efficiency is clearly seen. Δ79 and H2A.ddBbd nucleosomes were not

slided even under the highest concentration of RSC (Figure IV.5B and C). As previously, the results on H2A.ddBbd nucleosomes were verified by AFM analysis and no RSC induced nucleosome mobilization was seen on these nucleosomes (data not shown). We conclude that RSC is more sensitive than SWI/SNF to perturbations in the nucleosome structure resulting from defects in the docking domain of H2A.
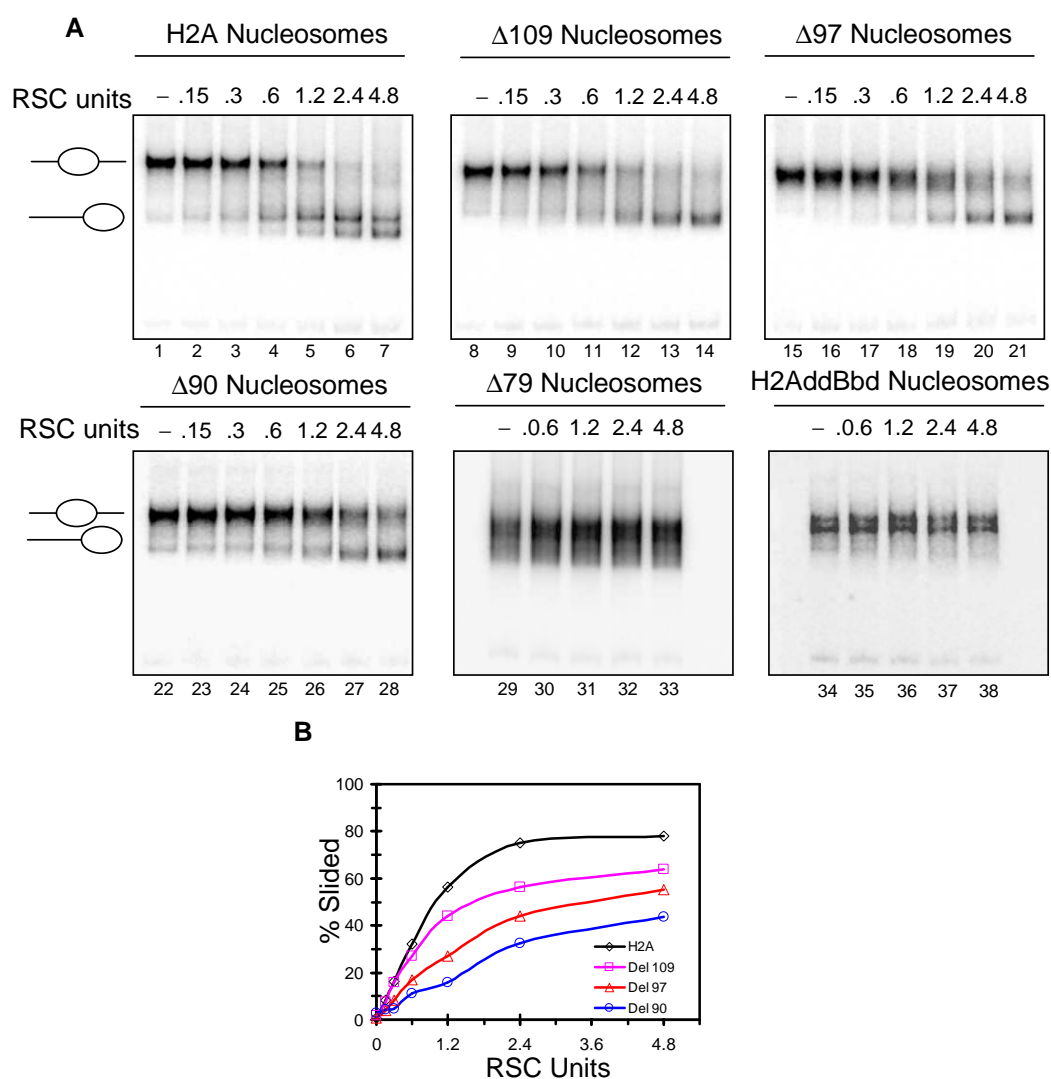
Figure IV.5. RSC is more sensitive to perturbations in the docking domain of H2A for nucleosome mobilization. (A) Centrally positioned conventional H2A (upper row left panel), Δ109 (upper row middle panel), Δ97 (upper row right panel), Δ90 (lower row left panel), Δ79 (lower row middle panel) and H2A.ddBbd (lower row right panel) containing nucleosomes on a 255 bp 601 DNA were incubated with increasing amounts of RSC (as indicated) in presence of 1mM ATP for 45 minutes at 29° C. Lanes 1, 8, 15, 22, 29 and 34 represent control reactions for respective nucleosomes without added RSC. Reactions were stopped by addition of 0.01 units of apyrase. Samples were resolved on 5% native PAGE. Gels were dried and visualized by exposure on a PhosphorImager. Positions of unmobilized and slided nucleosomes in the gel are shown by cartoon drawing. **(B)** Quantitation of gel data for conventional H2A, Δ109, Δ97 and Δ90 nucleosomes presented in A.

IV.2.5 Generation of distinct remodeled forms by SWI/SNF on H2A.Bbd nucleosomes

Recently, we have described a two step mechanism for SWI/SNF and RSC mediated sliding of nucleosomes where the first step is generation of remosomes characterized by having ~30-40 bp of DNA pumped in and distinct restriction enzyme accessibility profile of the nucleosomal DNA without translational repositioning (See Results chapter II & III). This step is followed by second binding of the remodeler complex and ATPase activity leading to mobilization of nucleosomes to the end of the DNA fragment. Therefore, inhibition of nucleosome sliding by incorporation of H2A.Bbd into nucleosomes can happen at either at the remosomes formation or the subsequent step of sliding. To dissect this issue we took advantage of the 'One pot restriction enzyme assay' (Wu and Travers, 2004). Note that, no translational repositioning was observed due to remodeler action on H2A.Bbd and H2A.ddBbd nucleosomes (Angelov et al., 2004; Figure IV.3, 4 and 5A). This allowed us to probe the true DNA accessibility (without the contribution of nucleosome repositioning) of remodeled nucleosomes in solution without the need for gel fractionation.

Briefly, we reconstituted H2A.Bbd containing nucleosomes on an equimolar mixture of 8 different 601.2 mutants containing *HaeIII* restriction site at different super helical locations (described in *materials and methods*). This allowed us to look at the accessibility of nucleosomal DNA with 10 bp resolution. 223 bp DNA fragments containing these sequences were PCR amplified and used for reconstitutions which position the nucleosome at one end of the DNA. Reconstituted nucleosomes were verified by gel shift assay and DNase I foot printing (data not shown).

Nucleosomes were remodeled in presence of SWI/SNF and ATP and the reaction was stopped by addition of apyrase. As a control, nucleosomes were incubated in presence of SWI/SNF but in absence of ATP. After stopping the reaction *HaeIII* was introduced in the reaction mixture to 5 units/µl and the restriction digestion was allowed to proceed. At indicated time points aliquots were taken and the reaction was stopped with addition of SDS and EDTA. DNA was extracted from the samples and resolved on denaturing PAGE. Figure IV.6A shows a representative experiment. Lanes 2-8 show the restriction enzyme accessibility profile of unremodeled H2A.Bbd nucleosomes. H2A.Bbd nucleosomes exhibit a characteristic accessibility profile. Last 3 superhelical locations d7, d6 and d5 (SHLs or dyads) are readily accessible to the restriction enzyme which is consistent with the previous observations where

146

the DNA ends in H2A.Bbd nucleosomes were shown to be less constrained (Doyen et al., 2006b; Bao et al., 2004). The accessibility drops suddenly from d4 to d1 and displays slow reaction kinetics (see Figure IV.6B for quantitation of the gel data). A characteristic feature of H2A.Bbd nucleosomes was the unusual accessibility profile at the dyad (d0). At this location the nucleosomal DNA seems to be highly dynamic and is accessible to restriction enzyme in a distinct manner. This is in agreement with the DNase I foot printing data where the most prominent perturbations were observed in the vicinity of the dyad (Figure IV.2). Note that, the restriction enzyme accessibility profile of these nucleosomes is completely different from canonical nucleosomes. In canonical nucleosomes maximum accessibility is seen at the end of the nucleosomes (i.e. d7) while d4-d0 are essentially inaccessible to the restriction enzyme (Chapter 3, Figure III.5). The picture, however, is changed when SWI/SNF remodeled nucleosomes are analyzed (Figure IV.6A lanes 9-15). The remodeled nucleosomes exhibit a peculiar accessibility profile where d7-d5 are largely unaffected. At dyads 2, 3 and 4 an initial jump in accessibility (leading to about 10-15 fold increase in accessibility) is observed after which it follows kinetics similar to that of unremodeled nucleosomes. At dyads 0 and 1 neither the accessibility nor the shape of the curve changes indicating that there is no effect of remodeling at this location.

The typical remosomes, as described previously, exhibit two characteristic features (i) A sharp decrease in accessibility at d7, indicating a strong attachment of pumped linker DNA inside the nucleosome and (ii) An overall increase in accessibility with the maxima at d0. Interestingly, none of these features were observed in SWI/SNF remodeled H2A.Bbd nucleosomes. Taken together, these results strongly suggest that the lack of nucleosome sliding observed in H2A.Bbd nucleosomes is due to a defective remosome formation.
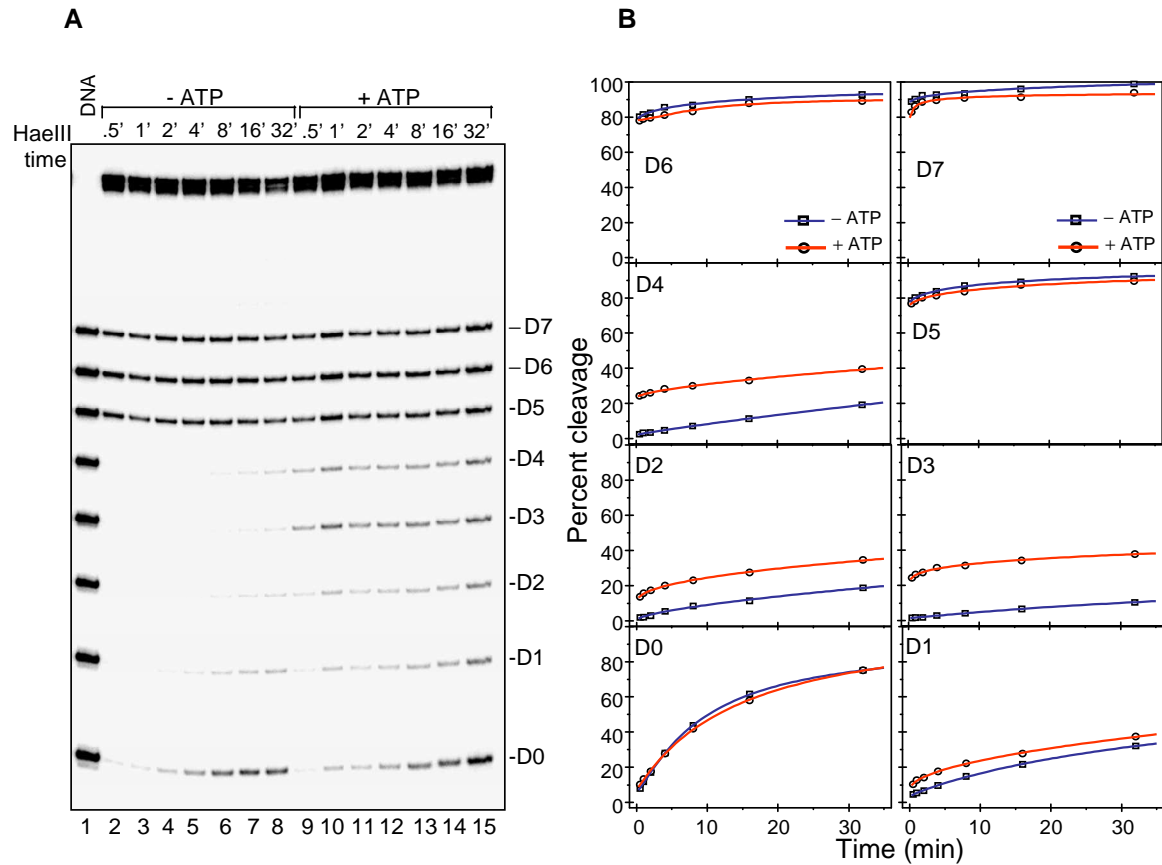
Figure IV.6. Generation of distinct remodeled forms by SWI/SNF on H2A.Bbd nucleosomes.

(**A**) H2A.Bbd nucleosomes were reconstituted by using the eight ^{32}P-labeled 223 bp 601.2 sequences, each containing a unique *Hae III* site (see material and methods for detail) and incubated with 2 units of SWI/SNF for 45 minutes at 29°C. After stopping the remodeling reaction by addition of apyrase, both control nucleosomes (incubated with SWI/SNF in the absence of ATP) and the SWI/SNF remodeled nucleosomes (incubated with SWI/SNF in the presence of ATP) were restriction digested with *HaeIII* (5units/μl). Aliquots were taken at indicated time points and reactions were stopped by adding SDS (0.1%) and EDTA (20mM). DNA was isolated, purified and run on 8% PAGE under denaturing conditions. Unremodeled (lanes 2-8) and remodeled (right panel lanes 9-15) nucleosomes, times of digestion with *HaeIII* and the positions of the different dyads are indicated. Free DNA, in the same condition, was digested for 1 minute (Lane 1). (**B**) Quantification of the data presented in (**A**). Kinetic curves for *HaeIII* accessibility are shown for unremodeled (in blue) and remodeled (in red) nucleosomes.

148

IV.2.6 The docking domain of H2A.Bbd is responsible for anomalous remodeling by SWI/SNF

The previous result of lack of characteristic remosomes formation on H2A.Bbd nucleosomes, also seen in lieu of available literature (Doyen et al., 2006b., Bao et al., 2004), strongly suggestive of the role of a defective docking domain. However, from previous experiment it can not be ruled out that whether the whole histone fold domain of H2A.Bbd is responsible for this behaviour. To test this, we performed similar assay with nucleosomes containing chimeric H2A where the docking domain was swapped with that of H2A.Bbd (H2A.ddBbd). A representative experiment is shown in Figure IV.7. Incorporation of this protein into nucleosome, expectedly so, ameliorates highly perturbed structure as seen with H2A.Bbd nucleosomes (Fig IV.7A lanes 1-7). At d7 (the end of the nucleosomes) the accessibility is very high as ~40 % of DNA at this dyad is cleaved within the first 30 seconds of HaeIII digestion and further goes to about 70% at 32 minutes (Figure IV.7B). However, unlike H2A.Bbd nucleosomes the accessibility of d6 and d5 is decreased and somewhat close to canonical nucleosomes (see Figure III.5). Another major difference is seen at d0 where histone DNA contacts seem to be greatly stabilized (compare d0 cleavage kinetics in figure IV.6B to that of 7B). The restriction enzyme accessibility profile of remodeled nucleosomes (figure IV.7A lanes 8-14) is, however, qualitatively very similar to those of remodeled H2A.Bbd nucleosomes. As with H2A.Bbd nucleosomes, SWI/SNF mediated remodeling resulted in increase in accessibility at d2, d3, and d4 with maximum at d3 (about 10-12 fold increase at d3 as seen in 30 seconds digestion with *HaeIII*). Additionally ~5 fold increase in accessibility at d5 was also seen at this time point. Note that this location DNA was highly accessible in H2A.Bbd nucleosomes and remained essentially unchanged after remodeling by SWI/SNF (Figure IV.6B). Importantly, no reduction in accessibility was observed at d7 and d6 as seen and rather a small increase was observed, indicative of lack of firm attachment of pumped DNA tightly associated with the octamer. Similar to H2A.Bbd nucleosomes, at d0 and d1 very little change in accessibility is seen (~ 2-3 fold as compared to ~10 folds with d3) at 30 second digestion of *HaeIII* (Figure IV.7B).

Taken together, these results clearly show that docking domain of H2A is essential for correct remosomes formation thereby affecting nucleosome mobilization by SWI/SNF.

Figure IV.7. The docking domain of H2A.Bbd is responsible for anomalous remodeling by SWI/SNF (**A**) SWI/SNF remodeling reaction was performed on H2A.ddBbd nucleosomes as described in Figure IV.6. Lanes 1-7 represent *HaeIII* digestion of control H2A.ddBbd nucleosomes (incubated in absence of ATP) at different time points. Similarly, lanes 8-14 represent *HaeIII* digestion of SWI/SNF remodeled nucleosomes. *HaeIII* concentration is kept similar (5 units/µl) as in Figure IV.6. Times of digestion with *HaeIII* and the positions of the different dyads are indicated. Free DNA, in the same condition, was digested for 1 minute (Lane 15). (**B**) Quantification of the data presented in (**A**). Kinetic curves for *HaeIII* accessibility are shown for unremodeled (in blue) and remodeled (in red) nucleosomes.

IV.2.7 RSC mediated remodeling is similar to SWI/SNF on H2A.dockingdomain.Bbd nucleosomes

Although SWI/SNF was not able to mobilize H2A.ddBbd nucleosomes, however, it was able to induce structural perturbations in the nucleosomes seen clearly in the restriction enzyme assay. The results of nucleosome sliding assays show that RSC is more sensitive to defects in the docking domain of H2A (Figure IV.5). This raised the question whether the initial remodeling process by RSC is also affected by these defects. To test this, we performed a similar one pot restriction enzyme accessibility assay. H2A.ddBbd nucleosomes were remodeled in presence of RSC and the accessibility of remodeled nucleosomes was assayed as described previously. Note that the activity of RSC was normalized with SWI/SNF by comparing its sliding activity on nucleosomes containing conventional H2A. A representative experiment is shown in figure IV.8. It is clearly seen that the RSC action on H2A.ddBbd nucleosomes gives rise to accessibility changes essentially similar to that of SWI/SNF (Figure IV.8A, compare lanes 1-7 to 8-14). The results were further confirmed when a quantitation of the accessibility of unremodeled and RSC remodeled nucleosomes was performed (Figure. IV.8B). Accessibility at different super helical locations of unremodeled H2A.ddBbd nucleosomes was compared to RSC remodeled nucleosomes at 16 minute time point of *HaeIII* digestion. A 2-3 fold increase in accessibility at d2-d4 was seen, consistent with the previous result of SWI/SNF mediated remodeling on these nucleosomes. As expected, accessibility at d0 does not change significantly. Moreover, no decrease in accessibility at d7 and d6 was observed, rather remodeling by RSC results in a small increase of accessibility at these super helical locations. We conclude that the first step of nucleosome remodeling by RSC is affected by a defective docking domain of H2A.
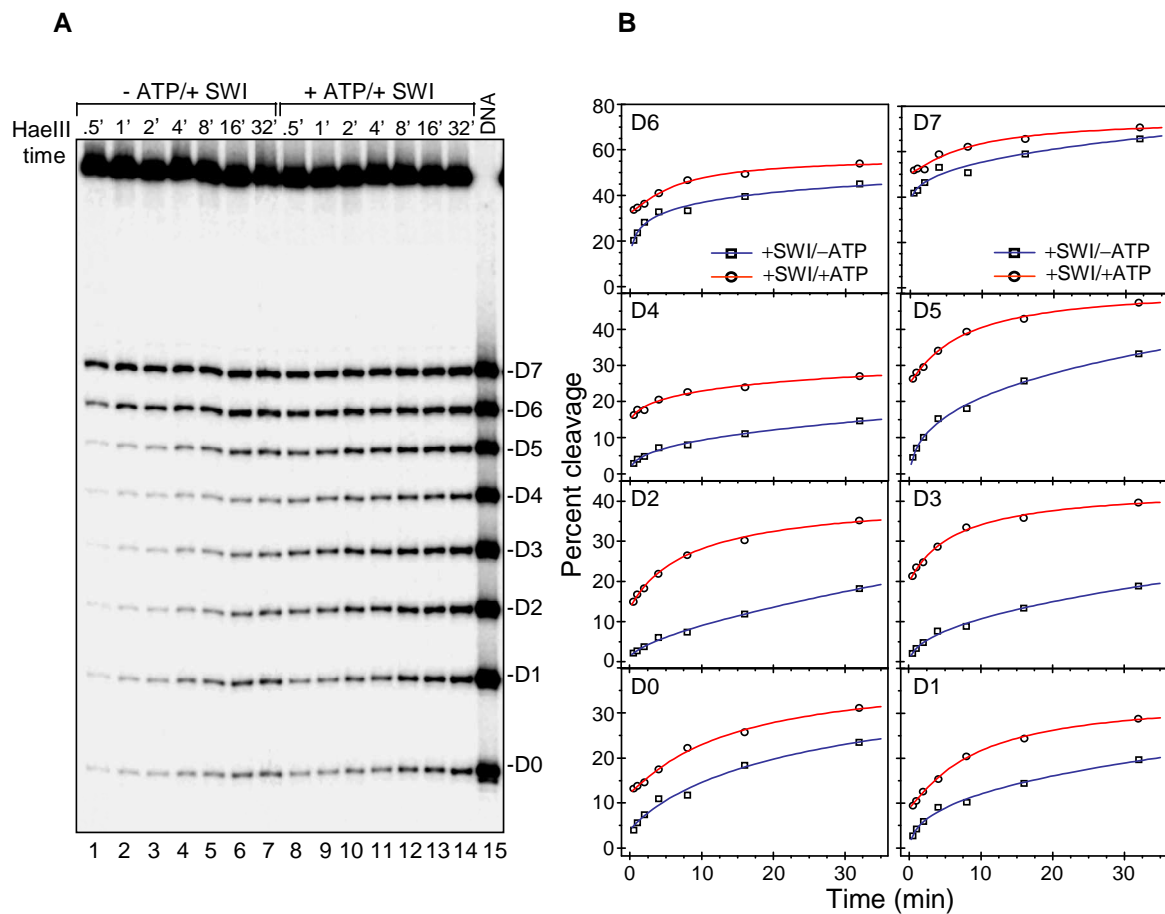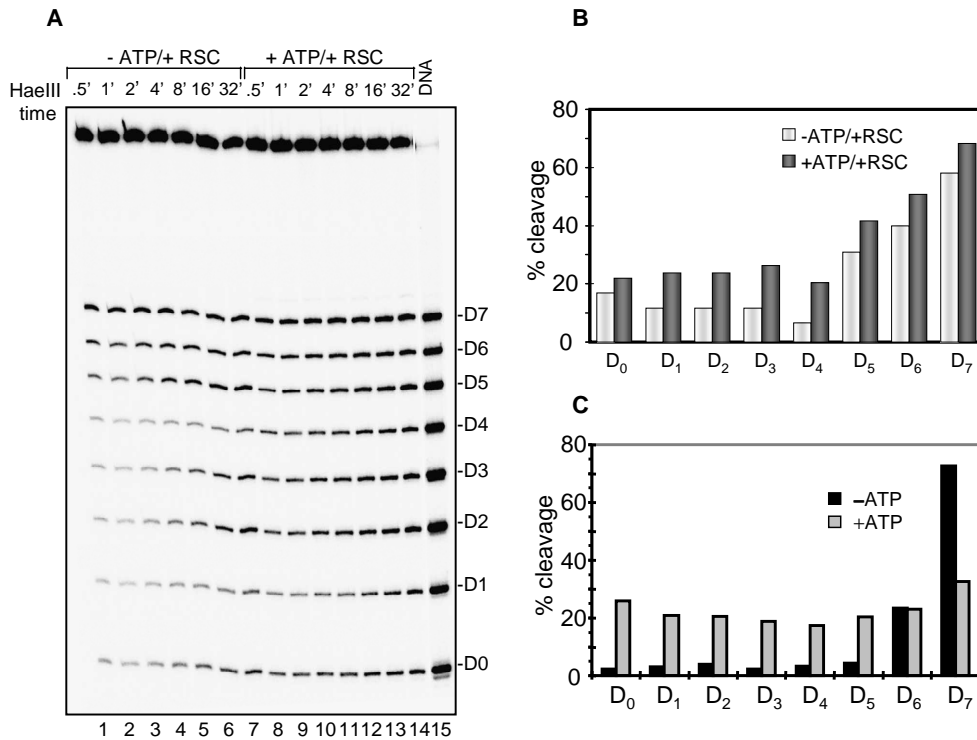
Figure IV.8. The docking domain of H2A.Bbd is responsible for anomalous remodeling by SWI/SNF (**A**) RSC remodeling reaction was performed on H2A.ddBbd nucleosomes as described in Figure IV.6. RSC activity was normalized to SWI/SNF as described in the text. Lanes 1-7 represent *HaeIII* digestion of control H2A.ddBbd nucleosomes (incubated in absence of ATP) at different time points. Similarly, lanes 8-14 represent *HaeIII* digestion of RSC remodeled nucleosomes. *HaeIII* concentration was kept at 5 units/µl. Times of digestion with *HaeIII* and the positions of the different dyads are indicated. Free DNA, in the same condition, was digested for 1 minute (Lane 15). (**B**) Quantification of *HaeIII* accessibility of unremodeled and RSC remodeled nucleosomes at 16 minute time point from (**A**). Light grey bars indicate unremodeled nucleosomes while dark grey bars represent remodeled H2A.ddBbd nucleosomes. Positions of respective dyads are denoted on x-axis. (**C**) Figure II.4C, lower right panel, reproduced here for comparison of accessibility profile of remosomes (from conventional nucleosomes) to that of remodeled H2A.dd.Bbd nucleosomes.

IV.3 Discussion

In the present work we have studied the role of H2A docking domain in nucleosome mobilization mediated by SWI/SNF and RSC. Nucleosome sliding assays using H2A C-terminal deletion as well the H2A.ddBbd chimeric proteins clearly demonstrated the importance of H2A docking domain in this process (Figure IV.3, 4 and 5). It is important to note that neither SWI/SNF binding nor ATPase activity is affected on H2A.Bbd nucleosomes (Angelov et al., 2004). The results presented here rather indicate towards an active structural role of histone octamer in chromatin remodeling process. SWI/SNF and RSC dependent remodeling of conventional nucleosomes starts with unwrapping and/or pumping DNA from

the linkers which is attached to the octamer forming a typical remosome structure. This, in turn, leads to decrease in the accessibility of dyads 7 and 6 and a concomitant increase in the accessibility at nucleosomal dyad (see results Chapter II&III). The situation is, however, completely different when the docking domain is defective, since no decrease is observed in the SWI/SNF or RSC remodeled H2A.Bbd and H2A.ddBbd nucleosomes (in the case of H2A.ddBbd nucleosome even a small increase in the accessibility of these dyads was detected) as shown by our 'one pot restriction enzyme assays' (Figure IV.6, 7 and 8). This suggests that the presence of a defective docking domain resulted in an inability to firmly attach the pumped extranucleosomal DNA on the octamer. The overall accessibility increase at all the dyads in the SWI/SNF H2A.Bbd and H2A.ddBbd remodeled nucleosomes evidences, however, that the remodeler is able to strongly perturb the histone-DNA interactions in these particles. Our AFM results are also in agreement to that (Figure IV.4B). These results are consistent with previous observation that SWI/SNF is able to increase accessibility of DNA on reconstituted $(H3-H4)_2$ tetramers arrays (Boyer et al., 2000) indicating that the $(H3-H4)_2$ tetramer is the minimal structural substrate for the first step remodeling i.e unwrapping and pumping of extranucleosomal DNA.

In our previous work (results chapter II and III), we have shown that nucleosome remodeling on canonical nucleosomes is a two step process where remosomes have been shown to be essential intermediates in the nucleosome mobilization process by SWI/SNF and RSC. Our data, presented in this study, clearly demonstrates that the formation is remosomes in nucleosomes lacking a correct docking domain is faulty and does not conform to typical remosomes structure. We believe that due to this the second round of binding and ATPase activity by SWI/SNF and RSC is non-productive and does not lead to nucleosome moblization.

We speculate that the inability of nucleosomes with a defective domain to firmly attach pumped DNA is due to a weakened H2A-H2B dimer and $(H3-H4)_2$ tetramer interface. Indeed, structural perturbations as seen by DNase I footprinting (Figure IV.2) and decrease in nucleosome sliding efficiency with RSC (Figure IV.5) were additive in nature and increased with progressive deletion of C-terminal H2A. These, together with the observations that H2A C-terminal truncations or incorporation of H2A.Bbd in nucleosomes weakens the H2A-H2B dimer and $(H3-H4)_2$ tetramer interaction (Eickbush et al., 1988; Doyen et al., 2006b) strongly suggest the role of H2A-H2B dimer in characteristic remosome formation. On the other hand,

the data presented here reinforces our proposed model where remosomes are essential intermediates during the nucleosome mobilization process by SWI/SNF and RSC.

IV.4 Experimental procedures

IV.4.1 Preparation of DNA probes

The 255 bp DNA probe was PCR amplified from pGEM-3Z-601 plasmid containing 601 positioning sequence in the middle (Kindly provided by J. Widom and B. Bartholomew). 5' end labeling was performed by using ^{32}P-labeled primer in the PCR. For 'One Pot Restriction enzyme Assay' a set of eight pGEM-3Z-601.2 mutants were used as a template, each containing HaeIII site at a different superhelical location, as described before (Wu et al., 2004). Briefly, a 223 bp fragment was amplified by PCR and 5' end labeling was performed. Labeling of the fragment was done as described above. For DNaseI and OH° footprinting a *NotI* restricted 601.1 fragment was 3' labeled using Klenow enzyme with [α-32P]CTP in the presence of 50 μM dGTP. All the DNA fragments were purified on 6% Native acrylamide gel prior to use for nucleosome reconstitutions. 255 bp cold 601.1 DNA was amplified using PCR for reconstitution of nucleosomes used in AFM experiments.

IV.4.2 Proteins

pET3a, containing HA tagged *Xenopus laevis* H2A between *NdeI* and *BamHI* sites was used as the parent clone for contsruction of H2A C-terminal deletion mutants. ORFs corresponding to HA-H2A Δ109, Δ97, Δ90 and Δ79 were PCR amplified and cloned into *NdeI* and *BamHI* digested pET3a vector (see supplementary methods for primer details). H2A.ddBbd chimeric protein was generated by primer overlap extension method (Constructed by Cecile Doyen). All the recombinant proteins including full length *Xenopus laevis* H2A, H2B, H3 and H4 were expressed in form of inclusion bodies in *E. coli* Strain BL21(DE3) and purified as described (Luger et al., 1999). Yeast SWI/SNF and RSC complexes were purified as described (Cairns et al., 1996; Côté et al., 1994).

IV.4.3 Nucleosome reconstitutions

Nucleosome reconstitution was performed by the salt dialysis procedure (Mutskov et al., 1998). Briefly, 2.4 μg Chicken erythrocyte Carrier DNA (200 bp average size) and 100ng of

either [32]P- labelled 255 bp 601, *NotI* restricted 601.1 fragment, or an equimolar mixture of 8 different 223 bp 601.2 mutant DNA fragments (100ng) were mixed with equimolar amount of histone octamer in Nucleosome Reconstitution Buffer (NRB) 2M NaCl, 10 mM Tris (pH 7.4), 1 mM EDTA, 5mM β MeEtOH. Reconstitutions with 255 bp unlabeled 601 DNA were also performed in the same way. In case of nucleosome reconstitutions with H2A deletion mutant or H2A.ddBbd proteins, H2A was replace by an equimolar amount of corresponding protein in the histone octamer. All the nucleosome reconstitutions were verified on 5% native PAGE run with 0.25X TBE.

IV.4.4 DNaseI and hydroxyl radical footprinting

150 fmol of nucleosomes, reconstituted on *NotI* digested 601 fragment, were digested with DNaseI in a volume of 7.5 µl buffer (10 mM Tris pH 7.4, 2.5 mM $MgCl_2$, 1 mM DTT, 100 µg/ml BSA, 50 mM NaCl, 0.01% NP40) for 2.5 minute at room temperature. Additionally 1 µg of plasmid DNA was added to the reaction mixture. DNaseI conditons for H2A and Δ109 were 0.14, 0.2 and 0.3 units. For other nucleosomes 0.9, 0.14 and 0.2 units of DNaseI were used. Reactions were stopped by adding 100 µl of 0.1% SDS and 20 mM EDTA. Hydroxyl radical footprinting was performed as described (Hayes and Lee, 1997). DNA was phenol:choloroform extracted, precipitated and run on 8% denaturing PAGE. Gels were dried, exposed and imaged on phosphorimager (Fuji-FLA5100).

IV.4.5 Nucleosome sliding assay

Nucleosome sliding reactions were performed with 150 fmol of nucleosomes in remodeling buffer (RB) 10 mM Tris pH 7.4, 5% glycerol, 1 mM rATP, 2.5 mM $MgCl_2$, 1 mM DTT, 100 µg/ml BSA, 50 mM NaCl, 0.01% NP40) in a volume of 7.5 µl at 29° C. The SWI/SNF and RSC units were defined as described before (Angelov et al., 2006). Nucleosomes were incubated with increasing amount of RSC or SWI/SNF for 45 minutes. Reactions were arrested by addition of 0.01 units of apyarse. Reaction products were resolved on 5% native PAGE. Gels were run in 0.25X TBE at room temperature and processed as described above. Sliding efficiency of indicated nucleosomes were calculated from quantitaion of gel scans.

IV.4.6 AFM analysis

For the AFM imaging, the SWI/SNF or RSC remodeled H2A.ddBbd nucleosomes were immobilized onto APTES-mica surfaces as described previously. Image acquisition and analysis were done as described in chapter II. DNA complexed length (L_c) and position (ΔL) distributions were constructed as described (Montel et al., 2007).

IV.4.7 One pot restriction assay

1 pmol of the H2A.Bbd or H2A.ddBbd nucleosomes on a mixture of 8 different 601.2 sequences (223 bp) were remodeled in presence of SWI/SNF or RSC in a volume of 42 µl in 0.4X restriction buffer (4mM Tris pH7.4, 0.4 mM DTT, 50 mM NaCl, 100µg/ml BSA) at 29° for 45 minutes . Only the remodeling reaction was supplemented with 1mM rATP while in control reaction no ATP was added. Amounts of SWI/SNF and RSC were scaled up proportionally (14 units). Reactions were arrested by adding 0.07 units of apyrase and HaeIII was added to 5 units/µl. Restriction digestion was allowed to proceed at 29° for indicated time points. Aliquots were taken and the reaction was stopped by addition of 0.1% SDS and 20 mM EDTA. DNA was extracted as described before and resolved on 8% denaturing gel. Gel scans were quantified using Multi-Gauge (Fuji). Data were normalized to the amount of radioactivity in each lane and % cleavage for each SHL (or dyads) were calculated and plotted against time of HaeIII digestion.

IV.4.8 <u>Supplementary information for Chapter II, III and IV</u>

<u>Wild type 601.2 sequence in pGEM-3Z</u>

CAGTGAATTGTAATACGACTCACTATAGGGCGAATTCGAGCTCGGTACCCTACATGCACA
GGATGTATATATCTGACACGTGCCTGGAGACTAGGGAGTAATCCCCTTGGCGGTTAAAAC
GCGGGGGACAGCGCGTACGTTCGATCAAGCGGATCCAGAGCTTGCTACGACCAATTGAG
CGGCCCCGGGACCAAGCTTCTGCAGGGCGCCCGCGTATAGGGTCCGGGGATCCTCTAGAG
TCGACCTGCAGGCATGCAAGCTTGAGTATTCTATAGTGTCACC

<u>Representation of HaeIII sites in the 601.2 sequences used for 'one pot assay'</u>

CAGTGAATTGTAATACGACTCACTATAGGGCGAATTCGAGCTCGGTACCCTACATGCACA

GGATGTATATATCTGACACGTGCCTGGAGACTAGGGAGTAATCCCCTTGGCGGTTAAAAC
 Dyad   Dyad1   Dyad2   Dyad3   Dyad4   Dyad5
GGCCGGGACAGGCCGTACGTGGCCTCAAGCGGCCCCAGAGGGCCCTACGAGGCCTTGAG
 Dyad6   Dyad7
CGGCCCCGGGAGGCCGCTTCTGGCCGGCGCCGGCCTATAGGGTCCGGGGATCCTCTAGAG

TCGACCTGCAGGCATGCAAGCTTGAGTATTCTATAGTGTCACC

<u>Oligos for 282 bp fragment :</u>

New_Trav_link_2[nd]: 5' CAGTGAATTGTAATACGACTC AC 3'

AT_Rev223: 5' GGTGACACTATAGAATACTCAAGC 3'

<u>Oligos for 223 bp fragment :</u>

AT_For: CAGGATGTATATATCTGACAC

AT_Rev223: GGTGACACTATAGAATACTCAAGC

<u>601.1WT (pGEM3Z-601):</u>

CTATCCGACTGGCACCGGCAAGGTCGCTGTTCAATACATGCACAGGATGTATATATCTGA
CACGTGCCTGGAGACTAGGGAGTAATCCCCTTGGCGGTTAAAACGCGGGGGACAGCGCG
TACGTGCGTTTAAGCGGTGCTAGAGCTTGCTACGACCAATTGAGCGGCCTCGGCACCGGG
ATTCTCCAGGGCGGCCGCGTATAGGGTCCATCACATAAGGGATGAACTCGGTGTGAAGA
ATCATGC

<u>Oligoes for 255 bp fragment:</u>

 601-Eco: GCTCGGAATTCTATCCGACTGGCACCGGCAAG

601-Bst: GCATGATTCTTAAGACCGAGTTCATCCCTTATGTG

Chapter V: General conclusions and perspectives

The mechanism of ATP dependent nucleosome remodeling has been a subject of numerous studies over the last decade. The basic outcome of chromatin remodeling is structural alterations in the nucleosome which facilitate access to factors involved in vital cell processes like replication, transcription, recombination and repair. The act of remodeling on nucleosomes results in at least 4 major outcomes (i) nucleosome sliding or movement of hostone octamer along the DNA in *cis*, (ii) removal of H2A-H2B dimers, (iii) nucleosome ejection i.e complete displacement of the histone octamer and (iv) replacement of H2A-H2B dimers by a variant histone like H2A.Z containing dimer (Cairns, 2007). Moreover, accessibility to factors can be generated through structural alteration in the DNA (nucleosome remodeling) around the histone octamer (Fan et al., 2004; Narlikar et al., 2004). Various models have been proposed for the nucleosome sliding, bulge propagation model being the currently favored model (Gangaraju and Bartholomew, 2007). However, no direct evidence of a bulge is presented. Moreover, the long standing question of generation of accessibility via nucleosome sliding or remodeling still remained unanswered as the experimental approaches used did not discriminate between a translational repositioning of the histone octamer or structural alterations.

The present study aimed at dissecting these issues by using a combinatorial approach of high resolution microscopy techniques and biochemical methods. We have identified, isolated and characterized novel intermediates of nucleosomes remodeling by RSC and SWI/SNF, two well characterized chromatin remodelers from yeast. These intermediates, termed remosomes, are peculiar structures which have distinct properties i.e. ~180-190 bp of DNA as compared to 147 bp in the canonical nucleosomes. An important feature of these particles was that despite of extra DNA pumped in side the nucleosomes no translational repositioning was observed through our AFM experiments. Moreover, the EC-M approach demonstrated that these particles do not represent a single well defined specie, but rather an ensemble of differently altered structures. Using biochemical techniques we were able to fractionate the remosomes as well as to visualize them by AFM. A very important feature of the remosomes was a distinct accessibility profile where the nucleosomal DNA was rendered accessible to a restriction enzyme all along the surface of the octamer. Further, we demonstrated that these remosomes are *bona-fide* intermediates of nucleosomes sliding process. The identification

of remosomes has allowed us, for the first time, to demonstrate the process of nucleosomes remodeling. Further, another major outcome of the study is the demonstration of the fact that nucleosome sliding is not a non-interrupted one step process but rather an iterative process going through the intermediary remosome generation.

We also addressed the issue of inference of nucleosome sliding by incorporation of histone variant H2A.Bbd in the nucleosomes. We demonstrated that a defective remosomes generation is the reason for this interference. Further we demonstrated that H2A docking domain is essential for nucleosome sliding by RSC and SWI/SNF through generation of characteristic remosomes. This observation also underscored our view that remosomes are essential intermediates in the nucleosome sliding process.

The identification of remosomes has raised many important questions. Are remosomes the structures responsible for the observed outcomes of ATP dependent nucleosomes remodeling like H2A-H2B dimer loss, exchange or whole octamer ejection? The biochemical evidence provided in our study strongly suggests that the interaction between the octamer and the DNA are highly perturbed. It is known that the tight wrapping of DNA is responsible for stabilizing the octamer and DNA interaction ((Luger et al., 1997; Bao et al., 2004)). Further analysis of stability of these particles would allow us to decipher this issue. Moreover, *in vivo*, these outcomes could be mediated through involvement of histone chaperones which could either destabilize or replace the H2A-H2B dimer with a variant histone containing dimer (Heo et al., 2008; Belotserkovskaya et al., 2003; Mizuguchi et al., 2004). Indeed, there is evidence that histone chaperones like Asf-1 can destabilize nucleosomes though interaction with H3-H4 tetramers *in vivo* and *in vitro* (English et al., 2006; Natsume et al., 2007; Schwabish and Struhl, 2006; and Korber et al., 2006). Further, depending upon the temporal availability of specific histones variants, histone exchange could be facilitated by generation of remosomes owing to their highly perturbed structure. We would like to test these hypotheses using purified remosomes and testing them as the source of histone related transactions in nucleosomes.

Generation of accessibility to factors without translational repositioning raises an attractive possibility *in vivo* scenario where generation of remosomes could help in overcoming nucleosome collision which is expected if nucleosome sliding is considered as the major outcome of ATP dependent remodeling by RSC and SWI/SNF. Furthermore, since no

translational repositioning is required for generation of remosomes, it could help in maintaining the positional memory for the nucleosomes while still allowing factor access to nucleosomal DNA.

Probably, the most interesting property of remosomes observed here is the random distribution of accessibility. This feature can be especially important for repair of DNA lesions encountered due to ionizing radiations or reactive oxygen species generated through the cell metabolism itself which are random in nature. It is established that organization of DNA into nucleosomes poses a strong barrier to these processes (Menoni et al., 2007). The inherent random distribution of factor accessibility of remosomes could help in overcoming this problem and possibly represent a major way of DNA repair *in vivo*. One may imagine that stochastic generation of remosomes is a necessary step for initiating global genome repair (GBR) by facilitating the initial recognition and binding of DNA glycosylases, the first enzymes in base excision repair. We plan, at least for the moment, to study the role remosomes in repair by a series of *in vitro* experiments.

In addition, we will also study how transcription factors can invade the nucleosome. The expectation is that within the remosomes, in contrast to conventional nucleosomes, the histone octamer would become "invisible" for transcription factors, i.e. the transcription factors would be able to invade the remosome with affinity very similar to that of naked DNA. If this is the case, the generation of remosomes would be a key factor in transcriptional regulation.

Are remosomes also formed upon nucleosome remodeling by other remodelers, belonging to the three other families, different from that of SWI/SNF family? If yes, what are their structures? Are they different or very close to those of the SWI/SNF and RSC generated remosomes? Do histone chaperones or other proteins with co-remodeling activity affect remosome formation? If yes, how do they do this?

The discovery of remosomes has presented a multitude of question of which only a part were enumerated above. Addressing these questions remains a challenge for future studies.

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164

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175

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