FACT, Base Excision Repair and Transcription Factor NF-kB binding to chromatin

John Lalith Charles Richard

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THÈSE

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Présentée par

John Lalith Charles Richard

Thèse dirigée par **Stefan DIMITROV** et
Codirigée par **Dimitar ANGELOV**

Préparée au sein du Laboratoire
Biologie Moléculaire et Cellulaire de la Différenciation
Unité INSERM U823
Institut Albert Bonniot
Université Joseph Fourier-Grenoble 1

Dans **l'École Doctorale Chimie et Science du Vivant**

FACT, Réparation par Excision de Bases et fixation du facteur de transcription NF-kB sur la chromatine

Thèse soutenue publiquement le 26 Juin 2012,
devant le jury composé de :

Dr. Pablo RADICELLA
CEA Fontenay-aux-roses — Rapporteur

Dr. Patrick LOMONTE
Université Claude Bernard Lyon — Rapporteur

Dr. Pierre COUBLE
Université Claude Bernard Lyon — Examinateur

Prof. Hans GEISELMANN
Universite Joseph Fourier — Examinateur

Dr. Dimitar ANGELOV
ENS de Lyon — Co-directeur de thèse

Dr. Stefan DIMITROV
Institut Albert Bonniot — Directeur de thèse

My Father: Late Mr Jacob Charles Philip Prasad
My Mother: Esther Rani Charles
My Brother: Charles Jacob Harrys Kishore
My Guide: My Lord and Saviour Jesus Christ

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Thesis Details

A. Title

a. Titre en Français

FACT, Réparation par Excision de Bases et fixation du facteur de transcription NF-κB sur la chromatine

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FACT, Base Excision Repair and Transcription Factor NF-κB binding to chromatin

B. Abstract

Résumé en Français

FACT est une protéine clé, qui joue de multiples rôles, y compris dans la transcription et la réparation de l'ADN endommagé. Néanmoins, comment FACT participe à la réparation et à la transcription de la chromatine n'est pas élucidé. Dans ce travail nous avons tout d'abord étudié le rôle de FACT dans le processus de réparation par excision de base (BER). Nous avons utilisé des nucléosomes reconstitués avec de l'ADN les thymines sont partiellement substitués avec de l'uracile. Nous avons trouvé que l'enzyme UDG est capable d'enlever les uraciles localisés du côté de la solution et pas les uraciles se trouvant en face de l'octamer d'histones. La présence simultanée de FACT et de RSC (facteur de remodelage de la chromatine, impliqué dans la réparation) permet un enlèvement efficace des uraciles localisés du côté de l'octamer d'histones par l'UDG. De plus, l'action concertée de FACT et RSC contribue à l'enlèvement de la lésion oxydative 8-oxoG, autrement inaccessible, de la matrice nucléosomale par l'enzyme OGG1. Ce résultat est obtenu grâce à une activité « co-remodelatrice » de la protéine FACT. Dans ce travail nous décrivons pour la première fois cette nouvelle propriété de FACT et nous montrons par une série d'expériences biochimiques que FACT est capable de stimuler l'activité de remodelage du RSC. Nos expériences montrent que la présence de FACT augmente l'efficacité de RSC à transformer l'énergie libérée par l'hydrolyse de l'ATP en travail « mécanique ». Les données obtenues suggèrent une nature stochastique du BER in vivo, FACT étant un facteur clé dans le

processus de réparation. Nous avons également investigué l'implication de l'activité co-remodelatrice de FACT dans la fixation de NF-κB aux matrices nucléosomales. La production de nucléosomes remodelés, mais non - mobilisés (remosomes) n'est pas suffisante pour promouvoir la fixation de NF-κB. Pourtant, la mobilisation des nucléosomes par l'intermédiaire de RSC permet une interaction efficace entre NF-κB et l'ADN nucléosomal. Toutes ces données sont essentielles pour le décryptage du mécanisme moléculaire par lequel FACT agit dans le BER et dans la transcription médiée par NF-κB.

Abstract in English

FACT is a vital protein which plays multiple roles in several processes including both transcription and repair of damaged DNA. However, how FACT assists repair and transcription remains elusive. In this work, we have first studied the role of FACT in Base Excision Repair (BER). We used nucleosomes containing DNA with randomly incorporated uracil. We found that the enzyme UDG is able to remove uracils facing the solution and not the uracils facing the histone octamer. The simultaneous presence of FACT and RSC (a chromatin remodeler involved in repair) allows, however, a very efficient removal of uracil facing the histone octamer by UDG. In addition, the concerted action of FACT and RSC permits the removal of the otherwise un-accessible oxidative lesion 8-oxoG from nucleosomal templates by OGG1. This was achieved by the co-remodeling activity of FACT, a novel property of this protein that we have discovered and analysed. The experiments reveal that the presence of FACT increases the efficiency of RSC to transform the energy released by ATP hydrolysis into "mechanical" work. The presented data suggest a stochastic nature of BER functioning *in vivo*, with FACT being a key factor in the repair process. The implication of the co-remodeling activity of FACT in NF-κB factor binding to nucleosomal templates was also investigated. The generation of remodeled, but not mobilized nucleosomes (remosomes), was not sufficient to promote NF-κB binding. However, the RSC-induced nucleosome mobilization allows efficient NF-κB interaction with nucleosomal DNA. Our data are instrumental in deciphering the molecular mechanism of FACT implication in BER and NF-κB mediated transcription.

C. Keywords

a. Mots clés en Français

Remodelage de la chromatine, nucléosome, FACT, RSC, UDG, chromatine, réparation, ADN, NF-κB

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D. Details of the laboratories of thesis preparation

a. Laboratory of affiliation

Unité INSERM U823

Institut Albert Bonniot

Domain de la Merci

38706 La Tronche cedex

FRANCE

b. Other Laboratory

Laboratoire de Biologie Moléculaire de la Cellule

UMR5239-ENS de Lyon-CNRS

École Normale Supérieure

46 allée d'Italie

69364 Lyon cedex 07

FRANCE

List of Abbreviations

ACF	ATP-dependent Chromatin assembly and remodeling Factor
AFM	Atomic Force Microscopy
ALS	Amyotrophic Lateral Sclerosis
AP-site	Apurinic Site
ATP	Adenosine-5'-triphosphate
BAP	Bramha Associated Protein
Bbd	Barr Body Deficient
BER	Base excision Repair
bp	Base Pair
BRG	Brahma Related Protein-1
BSA	Bovine Serum Albumin
CENPA	Centromere Protein A
CHD	Chromodomain Helicase DNA-binding
ChIP	Chromatin Immuno Precipitation
CHRAC	CHRomatin Accessibility Complex
CK2	Casein Kinase 2
COPD	Chronic obstructive Pulmonary Disorder
COX2	Cyclooxygenase 2
CS	Cockayne Syndrome
Da	Dalton (1g/mol)
DMS	Dimethylsuberimidate
DNA	Deoxyribo Nucleic Acid
DTT	Dithiothreitol
ECM	Electron Cryo Microscopy
EDTA	Ethylene Diamine Tetra Acetic acid
EM	Electron Microscopy
EMSA	Electrophoretic Mobility Shift Assay
ERK	Extracellular signal related Kinase
FACT	Fascilitates chromatin transcription
HAC	Histone Acetyltransferases
HAD	Histone deacetylases
Hae III	*Haemophilus aegypticus III*
HeLa	Henrietta Lacks
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HMG	High Mobility Group
hOGG1	human Oxoguanine Glycosylase
iNOS	Inducible nitric oxide Syntase
ISWI	Imititation SWItch
KDa	Kilo Dalton
LPS	Lipopolysaccharide
MAP	Mitogen Activated Protein
MBD	Methylcytosine Binding Domain

MTA	Metastasis associated Antigens
NaCl	Sodium Chloride
NCP	Nucleosome Core Particle
NER	Nucleotide Excision repair
NF-κB	Nuclear Factor Kappa-light-chain-enhancer of activated B cells
NHEJ	Non Homologous End Joining
nm	Nano meter
NURD	NUcleosome Remodeling and Deacetylation
NURF	Nucleosome Remodeling Factor
OH	Hydroxyl radical
PAGE	Polyacrylamide Gel Electrophoresis
PBAP	Poly Bramha Associated Protein
PCR	Polymerase Chain Reaction
PHD	Plant Homeo Domain
ROS	Reactive Oxygen Species
RSC	Remodels Structure of Chromatin
SDS	Sodium Dodecyl Sulfate
UDG	Uracil DNA Glycosylase
UV	Ultra Violet

1.1 Introduction

"The nucleic-acid 'system' that operates in terrestrial life is optimized (through evolution) chemistry incarnate. Why not use it to allow human beings to sculpt something new, perhaps beautiful, perhaps useful, certainly unnatural." **Roald Hoffmann, writing in *American Scientist*, 1994.**

Nature is a grandmaster when it comes to building one atom and one molecule at a time.
The master works of nature include well ordered, well structured materials be it the synthesis of materials like bones, teeth and corals or the more complex molecular machines that patrol the human body making biological processes like replication, signaling, transcription, translation and DNA repair. These exquisitely designed molecular machines have left us pondering at the mystery of how they function and how they are made in the first place.
In this thesis, we make an attempt at studying the dynamics and function of one class of these machines termed chromatin remodelers and the role of histone chaperone FACT (Facilitates Chromatin Transcription) in assisting their function in both nucleosome mobilization and transcription factor NF-κB binding.

1.2 Chromatin Structure, Organization and Dynamics

1.2.1 Chromatin

1.2.1.1 Landmarks in the field of Chromatin

In the nucleus the DNA exists as a complex structure called chromatin, which is composed of DNA and proteins (The word chromatin is derived from the Greek word "Khroma" meaning colored, based on its ability to stain with basic dyes). The term "Chromatin" was suggested for the first time by W Flemming. In the year of 1953, there was the discovery of the DNA double helix structure by Watson and Crick and two separate groups involving Wilkins and Franklin [1; 2; 3]. In the year of 1959, Zubay and colleagues were able to prepare soluble chromatin [4] and histones were fractionated by Johns [5]. With the advent of the electron microscopy H. Davies went on to report the chromatin threads that were 30nm in dimensions and were isolated from chicken erythrocyte nuclei [6]. In the following years Klug was able to purify these chromatin preparation and also suggested the solenoid model [7]. Olins and

Woodcock reported individually the 'beads on the thread' which the former called as v bodies [8; 9]. In 1974, Roger Kornberg in collaboration with J.Thomas postulated a model for the chromatin structure and in the subsequent year Pierre Chambon went ahead to coin the term Nucleosome for the chromatin subunit [10; 11]. With the advance in technology and the boon of X-ray crystallographic studies we were able to profit from the crystal structure of the nucleosome [12].

1.2.1.2 Chromatin and Chromosome Structure

The long strands of chromosomal DNA in the eukaryotic nucleus are approximately compacted 10000 to 50000 times in length. In spite of the tight packaging of the DNA the chromosome maintains its form and structure allowing regulatory proteins to access it, for transcription or replication. The molecular self-assembly takes place with DNA wrapping around "Histones". These histones are positively charged molecules that have a strong affinity towards the negatively charged DNA. When visualised under an electron microscope they appear as beads on a string every 200 bp [13]. Each bead is a nucleosome core particle that includes approximately 146 bp of DNA wrapped almost twice around a core histone octamer which comprises of a pair of each histone H2A, H2B, H3 and H4.

The Histone H1 (termed also as "linker" histone) binds to 40-70 bp of linker DNA, that separates adjacent core particles and helps compact the beads-on-a-string into fibers ~30 nm in diameter [7; 14]. When viewed under the electron microscope, these 30-nm fibers appear as helical structures with six nucleosomes per turn, an arrangement in which the DNA has been compacted ~40-fold in its linear dimension.

Laemmli and his colleagues [15; 16] took some stunning electron micrographs and provided enough insights into the organizations of the chromosomes. Their images mainly involved HeLa cell metaphase chromosomes stripped of histones showing DNA spooling out 30 to 90-kb loops from a proteinaceous "scaffold" that is the X shape of the paired sister chromatids [16]. If the histones were not stripped from the DNA then they appeared as loops of chromatin made up of approximately 180 to 300 nucleosomes coiled in the 30-nm fibre [15]. In cross section, the loops appear to radiate from the scaffold. Adjacent loop attachment sites are arranged in a helical spiral along the long axis of the metaphase scaffold [15]. Organizing 15 to 18 such loops per turn along the chromatid would account for ~1.2 million bp of DNA [17]. This arrangement probably allows the stacking of loops into a cylinder of chromatin

~800 to 1000 nm in thickness, which is in good agreement with the diameter of the metaphase chromosome[15; 17]. This model also accounts for the dimensions of metaphase chromosomes, which are ~10,000-fold shorter and 400 to 500-fold thicker than the double stranded DNA helices contained within them.

Figure 1 shows the step wise breakdown of the chromosomes into the chromatin and subsequently nucleosomes. On the right panel we see the two models of the 30nm fiber, the solenoid and the zigzag model.

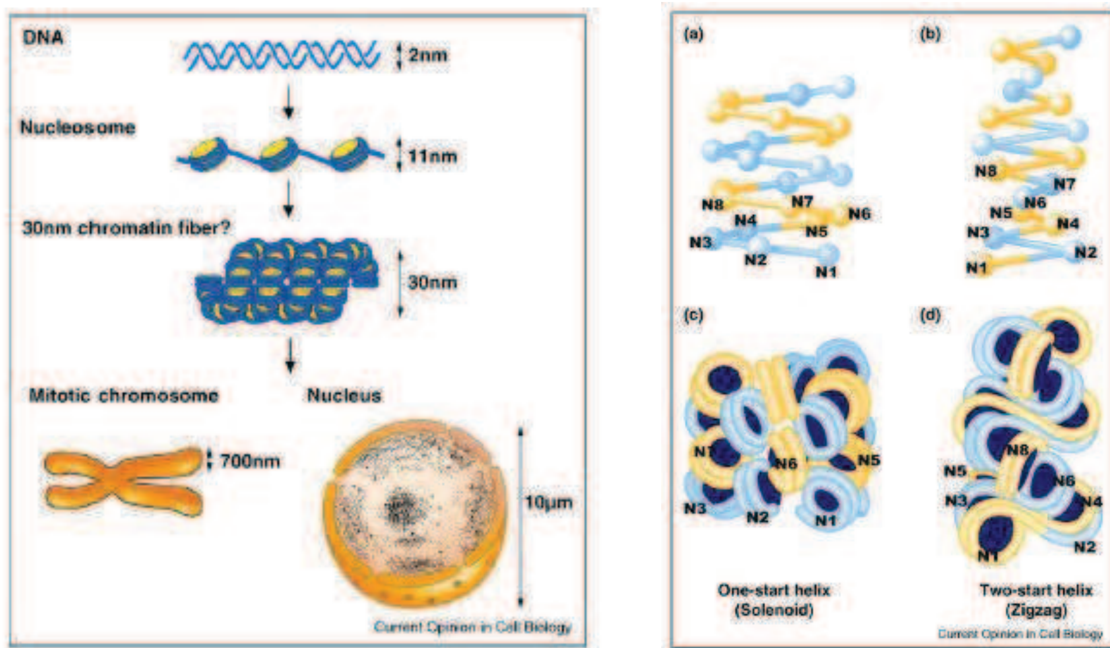

Figure 1: Chromatin organisation and chromatin architecture [18].

Chromosomes are divided into two distinct domains based on their structural and functional properties. The chromosomal regions that do not undergo post mitotic decondensation were termed as heterochromatin and the chromosomes that decondense and spread out in the interphase were referred to as the euchromatin [20]. The general properties of euchromatin and heterochromatin are summarised in Figure 2.

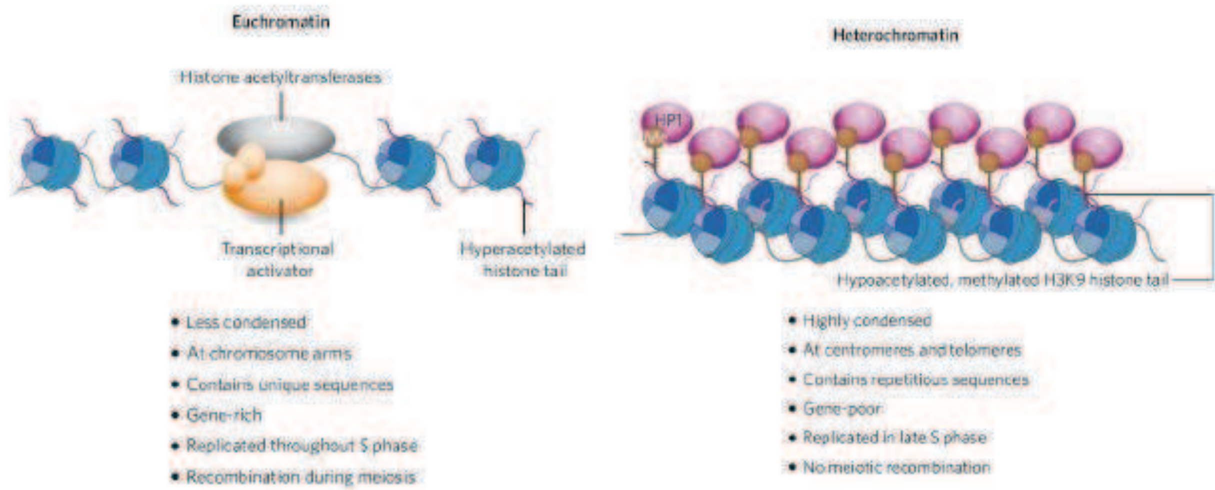

Figure 2: General properties of Euchromatin and heterochromatin[19].

1.2.2 The Nucleosome

The Nucleosome is the basic subunit of the chromatin and is interconnected by stretches of DNA called the linker DNA. Kornberg defined nucleosomes, as structures with about 200bp of DNA in close association with the core histones H2A,H2B, H3 and H4 [10]. In the absence of the linker histone the nucleosomes appear as beads on a string [8], but in the presence of the linker histone they condense and the linker histone binds at the entry and exit site of the DNA. This in a way helps in the compaction of the lengthy DNA strands. The nucleosome is formed in vitro by reconstituting the DNA and histones and a gradual step wise dialysis from 2M salt to 10mM salt is carried out. Figure 3 describes how the nucleosome is assembled, first by the deposition of tetramer and then followed by the dimer on the DNA fragment.

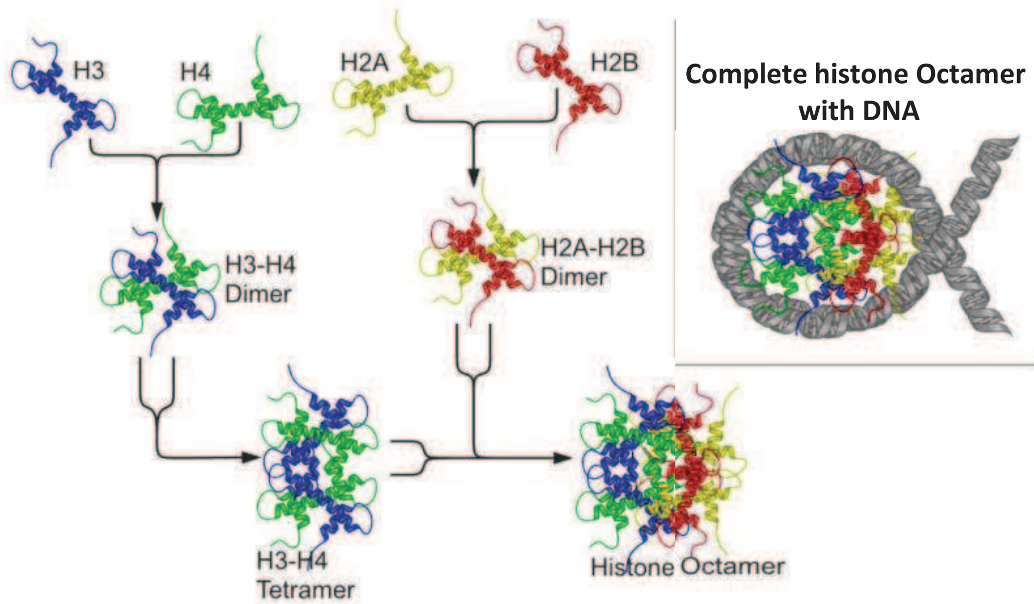

Figure 3: Assembly of Histones on the DNA to form the nucleosome.

1.2.3 The Histones

Histones were first described by A.Kossel in the year 1884 as an acid extractable material isolated from chicken erythrocyte. Histones are small basic proteins which are found in all eukaryotes and are highly conserved during evolution. There are 5 canonical forms of histones: H2A (14 kDa), H2B (14 kDa), H3 (15 kDa) and H4 (11 kDa) referred to as core histones and H1 (21 kDa) referred to as linker histone. The core histones have three domains namely the Histone fold domain, the N-terminal domain and accessory helices and less structured regions.

The histone fold domain consists of a helix-loop-helix motif. However, in the absence of DNA and in moderate salt conditions of 150mM, H3 and H4 form tetramers while H2A and H2B exist as dimers. In higher salt concentration of 2M an octamer of histones is formed. Apart from the histone fold domains there are the C-terminal and the N-terminal domains. The histone tails are prone to modifications and in turn are involved in the maintenance of the structure of chromatin. Linker histones help stabilize the higher order architecture. The linker histone does not resemble like the other histones and instead has a tripartite structure, made up of a globular domain flanked by a highly positive N and C terminal tails [21]. The

globular domain of histone H1 interacts with the entry and exit site of DNA in the nucleosome. Hydroxyl radical footprinting shows a particular signature when H1 is deposited on the nucleosomes [22].

1.3 Regulation of chromatin Dynamics

1.3.1 Replacement by Histone Variants

The nucleosome is a barrier to numerous vital cell processes that require access to free DNA [23]. The cell uses incorporation of histone variants, histone modifications and ATP dependant chromatin remodeling complexes to overcome this nucleosome barrier (see Figure 4) [24; 25; 26].

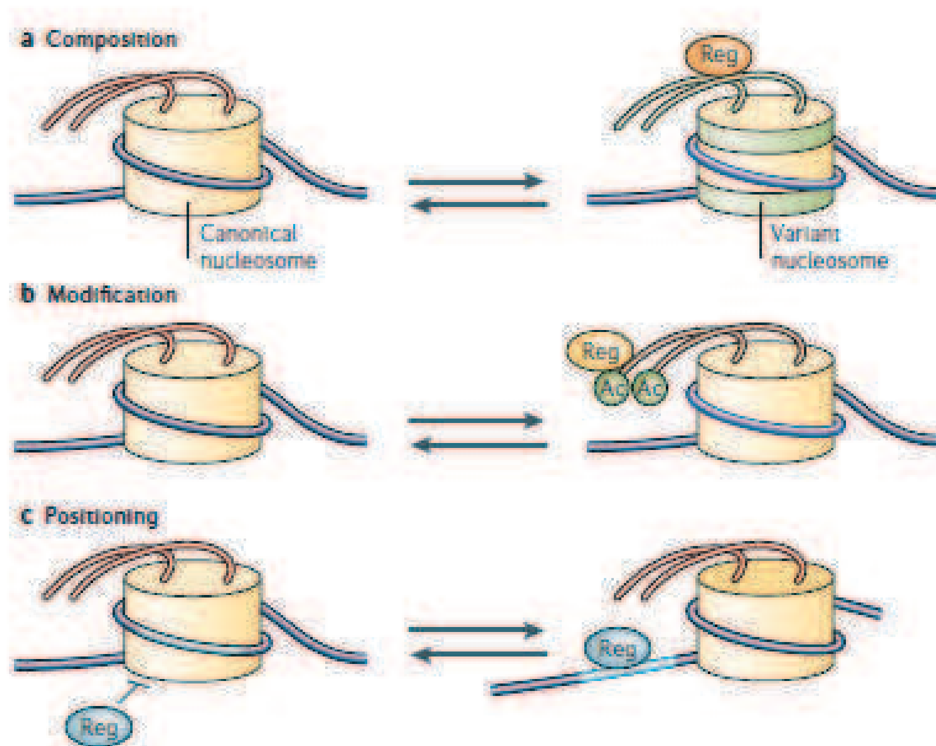

Figure 4: Nucleosome Dynamics: where (a) Incorporation of Histone variants (b) Modication of Histones and (c) chromatin remodeling. This image is adapted from a review on chromatin remodeling complexes [40].

The histone variants are non-allelic isoforms of conventional histones which are expressed in very low amounts in the cell [26]. In contrast to conventional histones, histone variants are incorporated into chromatin in a replication independant manner [26].

Multiple variants of all the core histones except histone H4 have been reported. The H2A and the H3 variants are the well studied variants. These variants exhibit a similarity ranging from 45- 90% [27; 28]. Previously it was reported that the carboxyl terminal of the H2A is essential for the stability of nucleosomal particles and that H2A-H2B and the tetramer interaction is hampered when the COOH terminus is truncated. Moreover, the remodeling and mobilization of the nucleosomes is reduced when the histone variants are incorporated [29; 30; 31; 32]. Some of the major histone variants of the histone H2A are H2A.X, H2A.Z, MacroH2A(mH2A) and H2A Barr Body deficient(H2A.Bbd) [33]. The major variants with the histone H3 which has been extensively studied are the H3.3 and the CENPA [34; 35]. The Figure 5 shows the conventional histones alongside the histone variants and their possible functions. The image is adapted from a review by Sarma and Danny Reinberg [36]. Incorporation of histone variants confers novel properties to the nucleosome [34]. Incorporation of histone variants and how they affect the main properties of the nucleosome is depicted in Figure 6. This image is adapted from a review on histone variants [34].

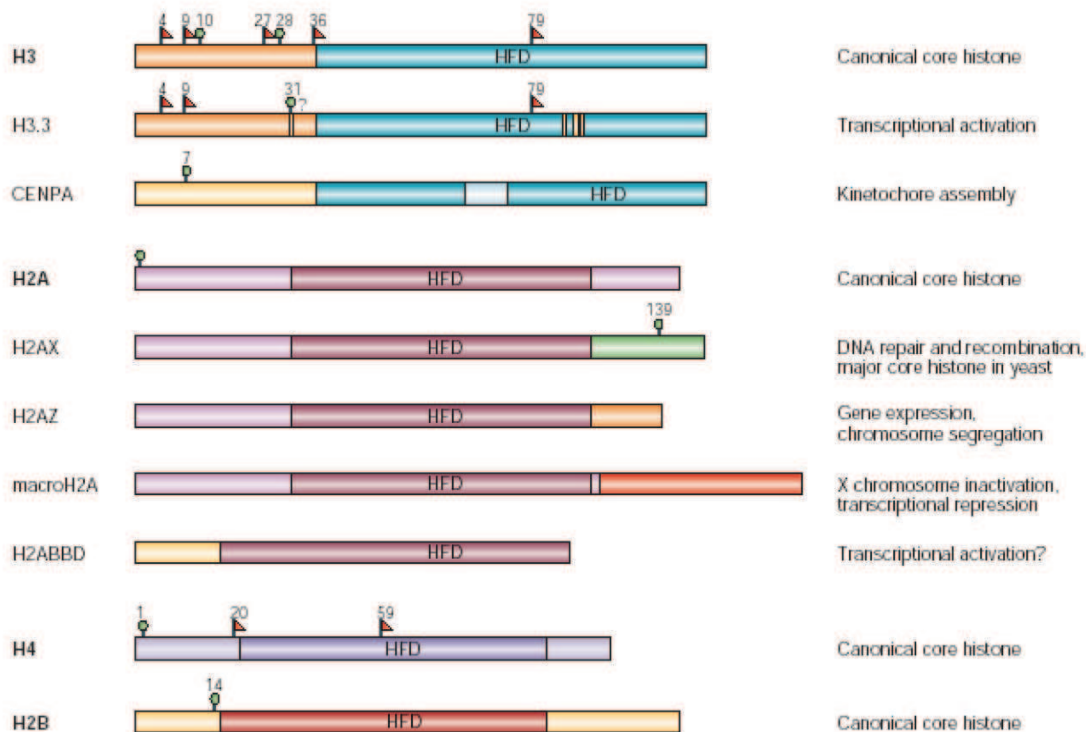

Figure 5: Histone Variants and their functions

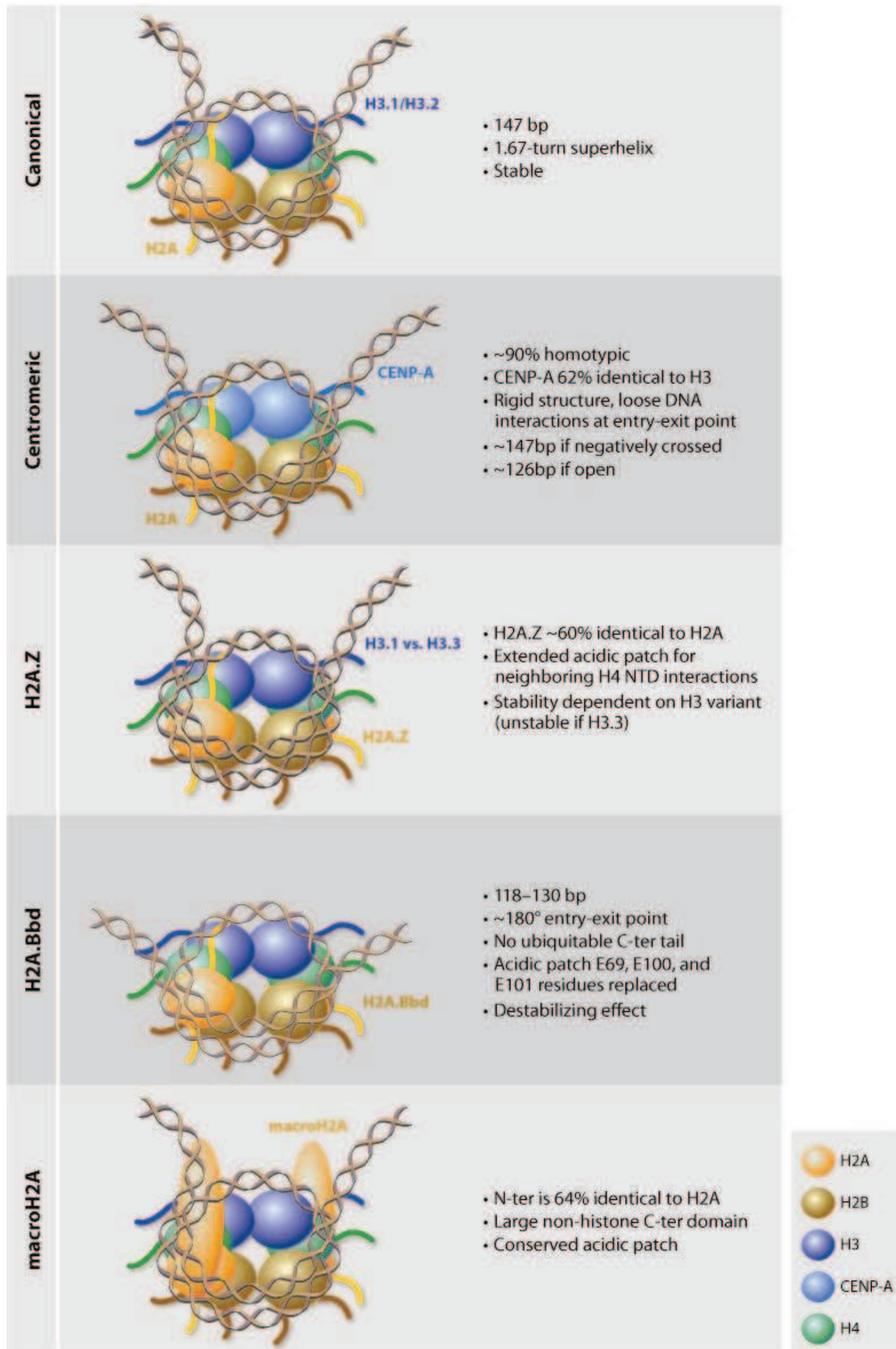

Figure 6: Incorporation of histone variants and the structural changes and perturbations incurred.

1.3.2 Post translational modification of histones

Chromatin modifications and gene regulations are an important branch of chromatin biochemistry. Core histones can be reversibly modified by acetylation, methylation, phosphorylation, ubiquitination or ADP-ribosylation and these modifications have consequences for gene activation, gene repression and chromosome replication. Lysines at the amino-terminal ends of the core histones are the predominant sites of known regulatory modifications. Active genes are preferentially associated with highly acetylated histones whereas inactive genes are associated with hypoacetylated histones. Histone acetylation and deacetylation are thought to exert their regulatory effects on gene expression by altering the accessibility of nucleosomal DNA to DNA-binding transcription activators, with the help of other chromatin modifying enzymes or multi-subunit chromatin remodeling complexes capable of displacing nucleosomes.

An example of the interconnections among histone modifications is the finding that deacetylation of lysine K9, located nine amino acids from the amino terminus of histone H3, is a prerequisite for methylation of this same lysine. Methylation of K9, in turn, recruits the binding of repressor proteins, such as HP1 (Heterochromatin Protein 1) that helps in establishing a highly compacted and transcriptionally inactive region of chromatin known as heterochromatin. The inter-connectedness of histone modifications that collectively influence a web of regulatory events has led to the hypothesis for a "histone code" controlling chromatin dynamics [24; 37; 38]. Such a code would allow post-translational modifications of various amino acids within the core histones to carry informational content and instructions that help specify which genes are to be activated or repressed.

In addition to modifying the histones that wrap the DNA into nucleosomes and higher-order structures, the DNA itself can be modified, most notably by the addition of methyl groups to cytosines. A high level of methylation is typically correlated with gene silencing, and is particularly evident in the silencing of transposable elements and multi-copy transgenes. A variety of DNA methyltransferases exists to modify the DNA in a variety of patterns. Some DNA methyltransferases act primarily in conjunction with replication to perpetuate methylation patterns from "mother" strands to newly synthesized "daughter" strands of a chromosome. Other DNA methyltransferases can add methyl groups to DNA strands that have no pre-existing methylation. Methylation of DNA may silence genes by preventing the binding of transcription factors. However, it is likely that cytosine methylation exerts most

negative effects on gene regulation via the involvement of other proteins that bind specifically to DNA when it is methylated. Indeed, a number of methyl-cytosine binding proteins have been identified and several are found in close association with one or more histone deacetylases. These findings suggest models, whereby cytosine methylation brings about local histone deacetylation, which could facilitate methylation of one or more deacetylated lysines on the histones and subsequent recruitment of repressor proteins that prevent transcription factors from gaining access to the given genes.

Some major protein domains involved with chromatin remodelling are as follows

- DNA methyltransferases (METs,CMTs,DRMs)
  These are the enzymes that methylate DNA in various patterns.
- Methylcytosine Binding Domain Proteins (MBDs).
  These proteins are thought to bind to methylated DNA to mediate other chromatin modifying events.
- Histone acetyltransferases (HACs).
  These enzymes add acetyl groups to histones.
- Histone deacetylases (HDAs).
  These enzymes remove acetyl groups from histones.
- Chromatin remodeling activities (CHR, CHB, CHC etc).
  These large multi-protein complexes use energy derived from the hydrolysis of ATP to alter the positioning of nucleosomes on DNA.
- SET Domain containing proteins (SDGs).
  SET domains are common within proteins that methylate histones.
- Chromodomain containing proteins.
  Chromodomains are found in histone-binding repressor proteins such as Heterochromatin Protein 1.
- Bromodomain containing proteins
  Bromodomains are found in proteins that bind acetylated lysines.
- High Mobility Group (HMG) Proteins
  HMG proteins are abundant non-histone chromosomal proteins that bind and bend DNA and provide for with "architectural" roles.

1.3.3 ATP dependant chromatin remodelling

The ATP dependant chromatin remodelers function either by repositioning nucleosomes, altering the nucleosomal conformations, changing histone compositions or ejecting histones from the DNA [39].

1.3.3.1 Chromatin remodeler Families

1.3.3.1.1 SWI/SNF Family of Chromatin remodelers

This family of chromatin remodelers have been named the SWI/SNF family mainly because of the discovery of the first member ySWI/SNF [41; 42; 43]. The yeast SWI/SNF complex consists of 11 subunits namely, SWI2/SNF2, SWI3, SNF5, SNF6, SNF11, SWP82, SWP73, SWP29, ARP7 and ARP9 [43; 44; 45; 46]. Most of the SWI/SNF components have similar homologous counterparts in other SWI/SNF like remodelers, thus suggesting some kind of conservation of function. For example, SWI1p shows homology to OSA and Baf250p of dBrahma and hSWI/SNFa. SWI/SNF like remodelers have a tendency to bind to naked DNA and to nucleosomal DNA in an ATP dependant manner [47; 48]. A striking similarity of these SWI/SNF remodelers is with High Mobility Group Box HMGB containing proteins in their ability to bind to DNA structures and not to sequence specific DNA [47; 48]. Moving towards higher organisms there is the presence of SWI/SNF like complexes in drosophila called BAP(Brahma Associated Protein) or PBAP (Polybromo associated BAP) [49]. Likewise there are at least two SWI/SNF like remodelers in humans, BRG1/ hSWI/SNFA or hPBAF or hSWI/SNFB. A snapshot of the SWI/SNF family members and their functions is illustrated in Figure 7.

RSC (Remodels Chromatin Structure)

RSC is 15 subunit 1MDa protein isolated from yeast [50]. The 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). This particular remodeler is available in plenty compared to SWI/SNF [50]. There are some isoforms of RSC purified by RSC6 antibody short of 90 KDa and missing subunits RSC3 and RSC30 and this was referred to as RSCa. There are two other isoforms, one containing RSC1 and the other RSC2. While RSC2 containing isoform is more abundant,

deletion of either of the genes leads to defects in growth and a double deletion is lethal [51]. RSC unlike SWI/SNF is involved in mitosis and in repair of damaged DNA.

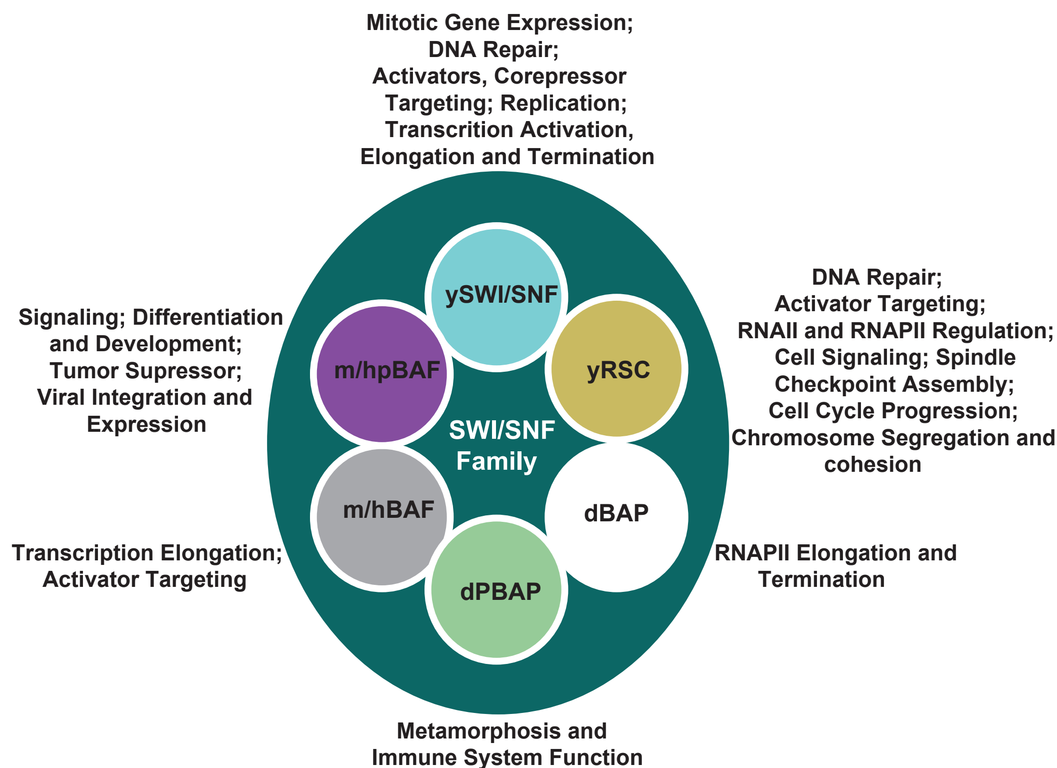

Figure 7: The SWI/SNF Family of remodelers and their functions.

1.3.3.1.2 ISWI family of Remodelers

This group of ATP dependant chromatin remodelers get their name from their similarity to the SWI2 ATPase and hence Imitation SWItch. This ATPase is highly similar to Brahma and was first discovered in drosophila [52]. ISWI family of remodelers have two SANT domains in the C-terminal region and do not have the bromodomain [53; 54]. They have a preference to binding to nucleosomes with extra linker DNA than to nucleosome core particles. The ISWI group of remodelers play an important role in transcription activation and repression as well. A homozygous null mutation of ISWI leads to lethality in drosophila [55]. This suggests that it plays an important role in gene expression. Apart from this it has also been shown to be involved in maintenance of higher order chromatin structure [55].

The ISWI complex is composed of 2-4 subunits and are about 200-800 kDa in size. dNURF and dCHRAC were first identified in drosophila [56; 57]. Drosophila contains three ISWI complexes namely, NURF (NUcleosome Remodeling Factor), ACF (ATP-utilising Chromatin Factor) and CHRAC (CHRomatin Accessibility Complex). The ATPase activity of these remodelers is activated by nucleosomes and not by DNA. dNURF interacts with the histone tail of H4 and helps mobilizing the nucleosomes [58]. ACF has been reported as a regulator of nucleosome spacing [59; 60]. ACF is also capable of sliding the end positioned nucleosomes to centre position [61]. CHRAC is closely related to ACF and can perform like ACF in spacing the nucleosomes and sliding them [57; 62]. Based on the homology with drosophila ISWI, Tsukiyama and colleagues indentified two ISWI genes ISWI1 and ISWI2 in yeast [63]. In higher eukaryotes such as *Xenopus laevis*, mouse and human there are ISWI complexes called RSF (Remodeling and Spacing Factor), hACF, WCRF(Williams Syndrome Associated Chromatin remodeling Factor), hCHRAC [64; 65; 66; 67; 68]. As in yeast two more genes in humans were identified and they were called hSNF2L and hSNF2H with almost 70% homology to the yeast genes [69; 70]. Figure 8 provides a summary of the ISWi family members and their general functions.

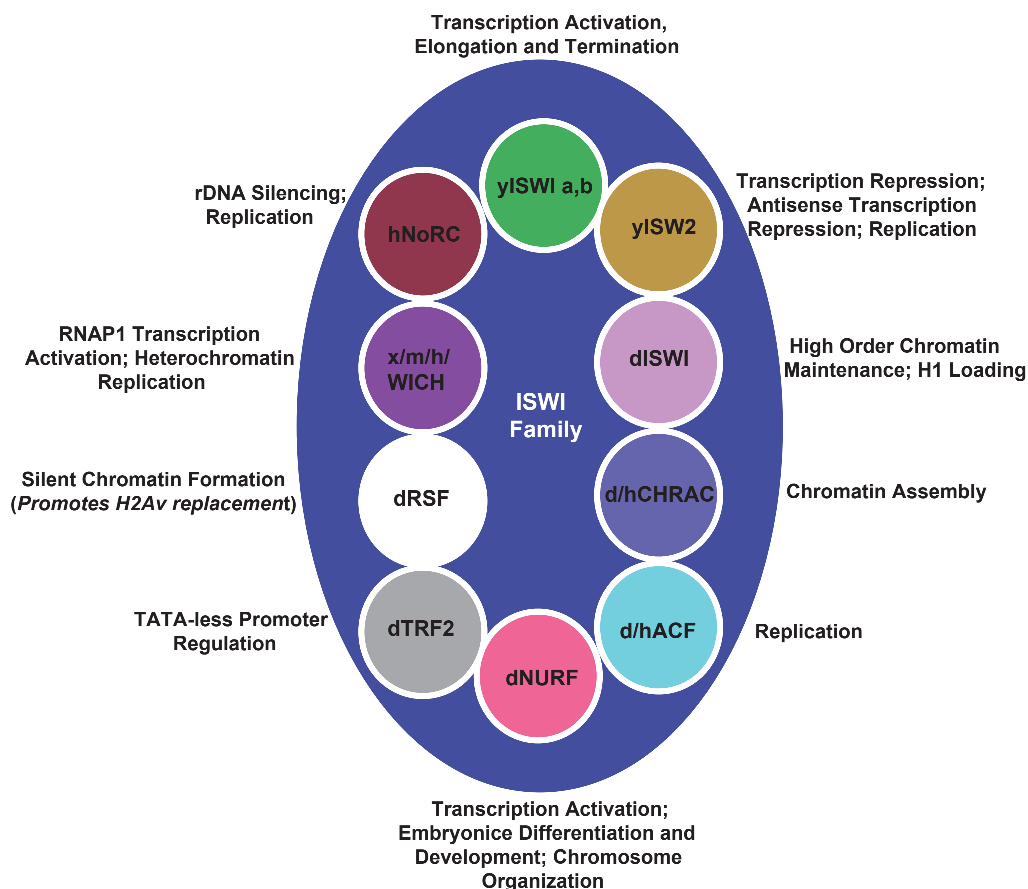

Figure 8: The ISWI group of remodelers and their functions.

1.3.3.1.3 CHD family of Remodelers

This particular group of remodelers play an important role in development. It has been shown that mutations in dMi-2 is lethal at the embryonic stage [71]. The CHD (Chromodomain Helicase DNA-binding) or Mi-2 complexes contain ATPases with one or more chromodomains. The first CHD protein was identified in mouse and was isolated as a protein which showed the properties of both the SWI2/SNF2 group of remodelers and Polycomb/HP1 chromodomain family of proteins. The different CHD remodelers and their roles in the cell are summarised in Figure 9.

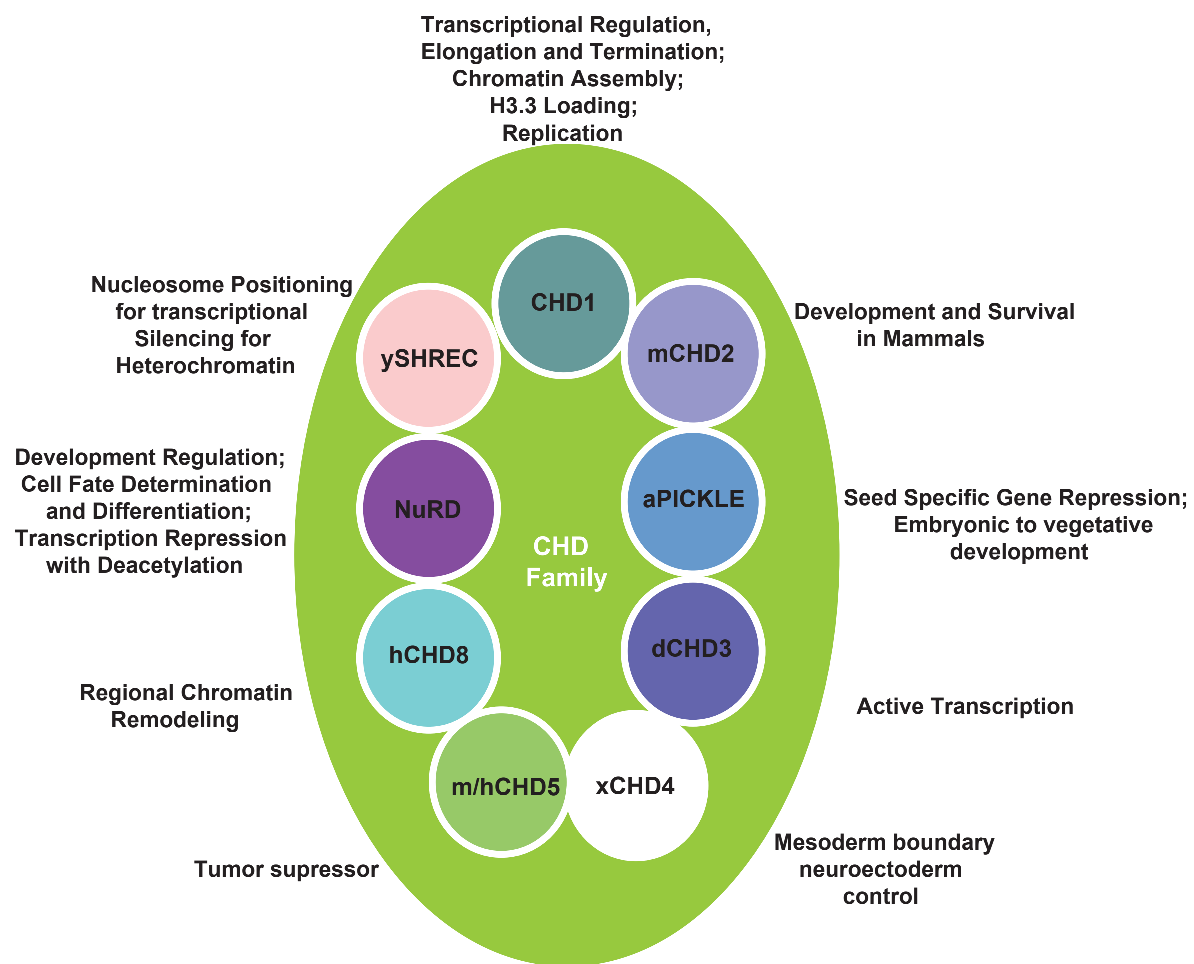

Figure 9: The CHD family of Chromatin Remodelers.

1.3.3.1.4 INO80 family of remodelers

This is again a large complex protein with 15 subunits and is known to be involved in DNA repair and transcription activation. One of the largest subunits INO80p has a conserved but discontinuous ATPase domain, with a huge spacer region in the middle. There are two conserved motifs TELY motif at the amino terminus and GTIE motif at the carboxy terminus [72]. There are also actin related proteins associated to this complex like Arp 4, 5 and 8. Rvb1 and Rvb2 are found to be present in multiple copies per INO80 molecule and also have a helicase activity. Yeast mutants of INO80 show an increased hypersensitivity to DNA damaging agents and also defects in regulation of transcription. Also the Nhp10 subunit of INO80 was reported to be recruited in double strand break repair [73; 74]. A similarly large complex called SWR1 was found with 14 subunits and almost 4 subunits similar to INO80 such as Rvb1, Rvb2, Act1 and Arp4 [75; 76; 77]. SWR1 has been reported to play a role in DNA repair and also is involved in replacement of H2A/H2B dimers with H2A.Z /H2B dimers in an ATP dependant manner [77]. The involvement in cell events by INO80 family of remodelers is schematically described in Figure 10.

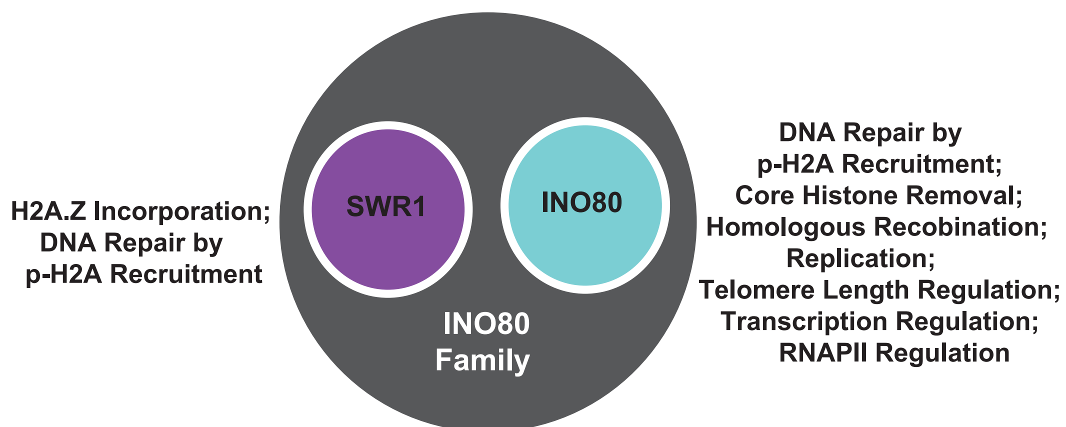

Figure 10: The INO80 family of remodelers.

1.3.3.2 Domains involved in Remodeler – Nucleosome Interaction

There are multiple remodeler domains that are involved in nucleosome recognition and interaction. However, it is not clear whether they act as individual domains or in concert with other domains. Some of the important and well characterised domains are as follows (See Figure 11).

1.3.3.2.1 Bromodomain

The bromodomain in the remodelers is distinct in its ability to recognise the acetylated lysines in the histones. In the case of SWI/SNF there exists such a bromodomain in the C-terminal region of the protein. Yeast, flies and humans all make two SWI/SNF related complexes and one of them has multiple bromodomains. These bromodomians can exist in a single protein called as polybromo or can exist in a number of proteins such as yRSC1/2/4/10 and open the possibility for cooperative recognition of modifications. Noteworthy, it has been reported that acetylated nucleosomes are easily mobilised by SWI/SNF [78; 79].

1.3.3.2.2 Chromo Domain (CHD)

The chromodomains is characteristic of the CHD family of remodelers. These remodelers have two chromodomains in their N terminal domains. These N-terminal domains behave as a structural unit to help the binding of the remodeler to a methylated lysine [80; 81; 82]. The presence of the chromodomains is highly necessary for the remodeler to both bind and remodel the nucleosomes [80].

1.3.3.2.3 Plant Homeodomains (PHD)

The Plant Homeodomain PHD finger has a methyl-lysine interaction motif. The PHD finger is reported to interact functionally with the bromodomains of the ISWI and the chromodomains of the CHD family remodelers [83]. However, in the case of dNURF the PHD finger of BPTF subunit interacts with the H3K4me3 stabilizing the BPTF/NURF interaction with active chromatin [84]. Apart from histone tails there are other epitopes for the recognition by the PHD domain. For example the PHD of dACF recognises the globular domain of the core histones [61].

1.3.3.2.4 Hand SANT-SLIDE Domain

Several remodelers possess Hand SANT-SLIDE domain.This domain consists of three sub-domains and is found in the C-terminal region of the ISWI remodelers. The SANT domain is also found in a lot of chromatin related proteins. The SANT is required for the function of ySWI/SNF and is present in ySWI3. Similarly this domain is necessary for the functioning of

the yRSC and is present in the yRSC8. It is present in yAda2 and enhances the yGcn5 HAT activity [85]. While the SANT domain interacts with the histones, the SLIDE domain contacts the nucleosomal DNA. However, when the SLIDE domain is deleted in the dISWI there is loss of the ATPase and remodelling activities, in the contrary when the SANT domain is deleted, then there is reduced remodelling [53]. Thus we can say that this particular domain is characteristic of the ISWI family of remodelers and that they cooperate with each other in nucleosome recognition and stimulation of ATPase activities.

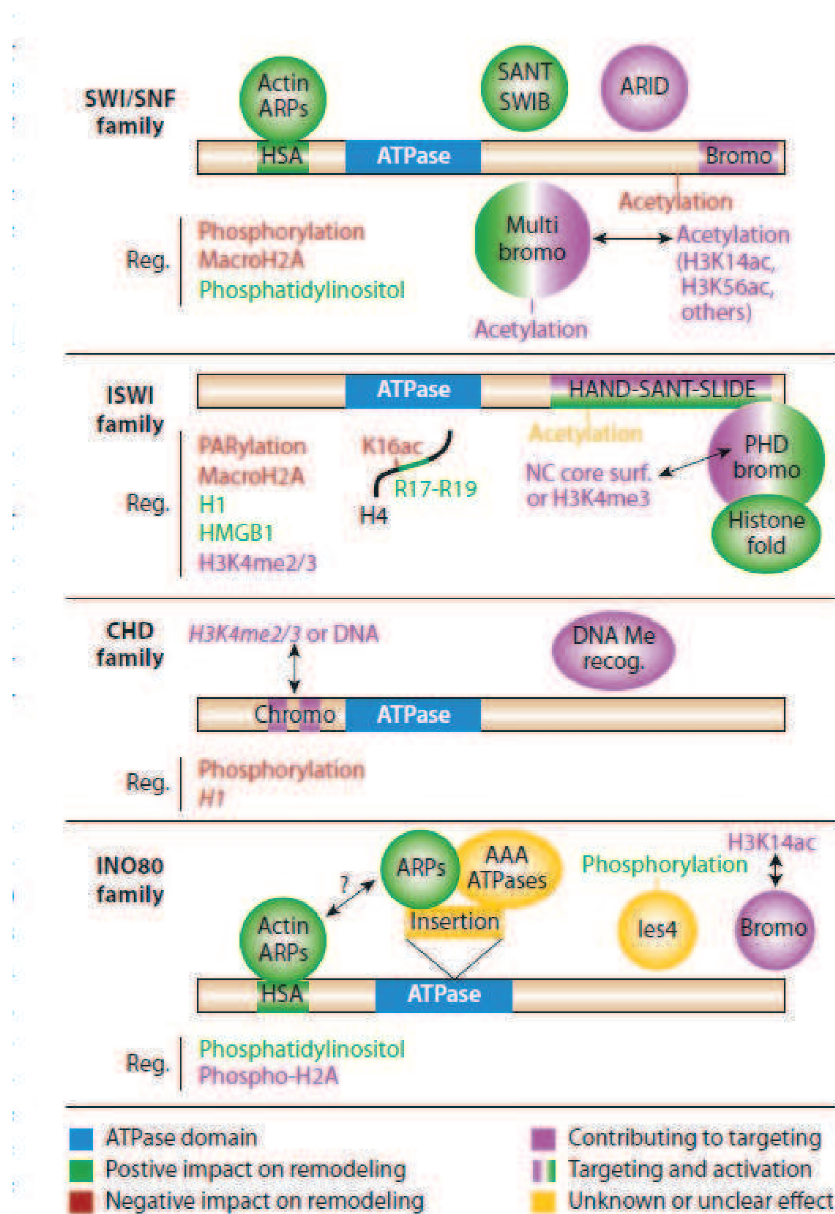

Figure 11: Chromatin Remodeler Domains The image is adapted from [86] and shows the various domains involved in the chromatin remodelers.

1.3.3.3 ATP dependant chromatin remodeling: Mechanism

The remodelers alter the chromatin structure making them more accessible to the DNA for processes like transcription and repair. In this part of the manuscript I will briefly summarize the data on the mechanism of nucleosome remodeling by the different remodelers.

1.3.3.3.1 Substrate Binding

The remodelers have the tendency to recognise the nucleosomes as their substrates. However, they interact both with the DNA and the histone part of the nucleosomes during their activity. In the beginning DNA affinity columns were used for the isolation of SWI/SNF [87]. The binding of the remodelers to the nucleosomal substrate was previously shown by band shift experiments [44]. The SWI/SNF remodelers show an affinity for DNA substrates in an ATP dependant fashion. In the case of ISWI remodelers there is lower affinity in binding with DNA in comparison with SWI/SNF remodelers [88]. There are also instances where the linker DNA helps in the remodelers to bind with the nucleosome [89].

Evidence for the same is provided by cross-linking experiments [90] and electron micrographs [91; 92; 93; 94]. There are distinct places where the DNA and the histones in the nucleosomes come in contact with each other and the remodeler [40; 91; 95]. Leschziner used Orthogonal Tilt Reconstruction method (OTR) and showed that RSC posses a deep central cavity sufficient to fit in one complete nucleosome [94] (see Figure 12).

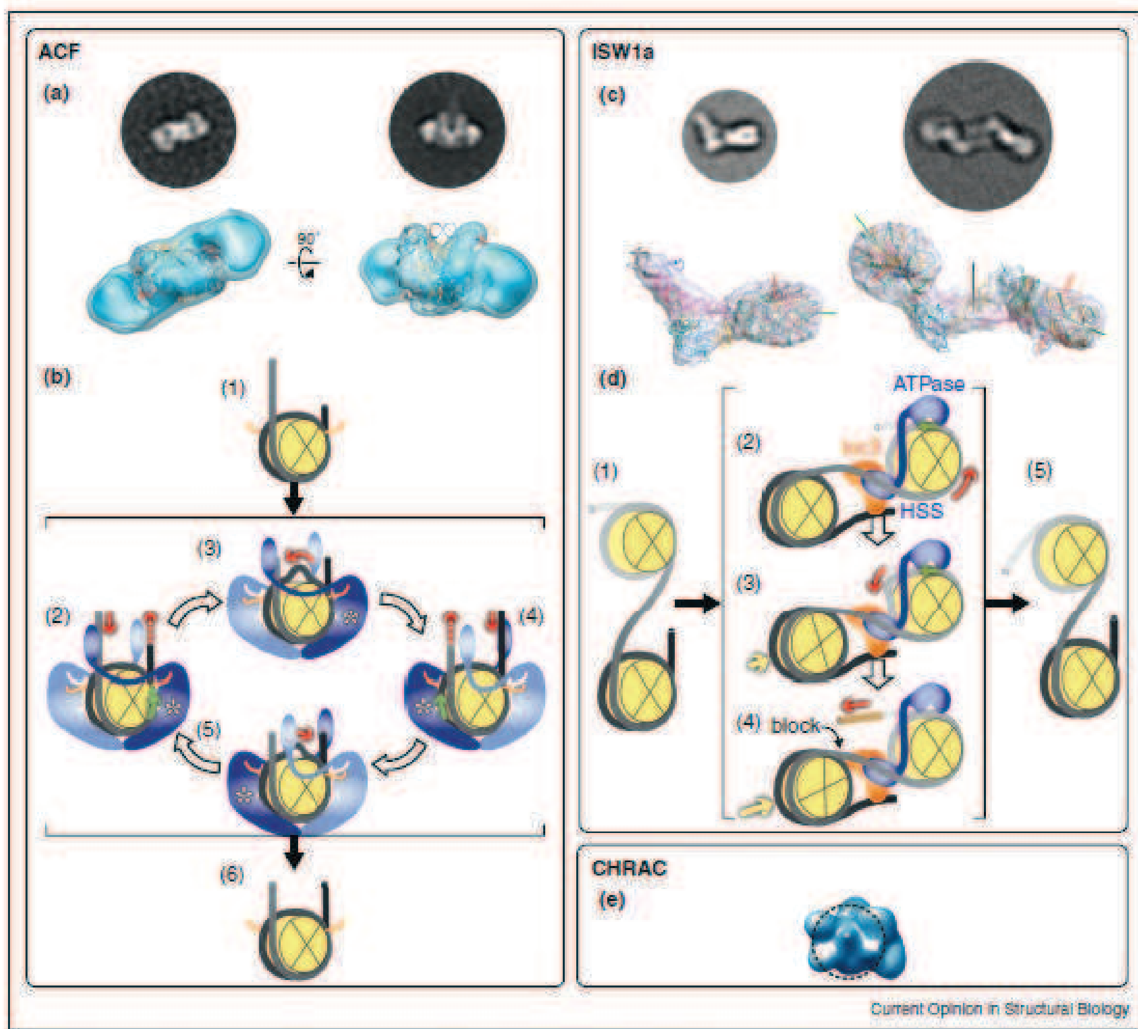

Figure 12: Electron microscopy reconstructions of ISWI class of remodelers

1.3.3.3.2 ATP binding and Hydrolysis

The remodelers require ATP to carry out its functions of altering nucleosome structures. In case of SWI/SNF class of remodelers, their ATPase activity is stimulated by single stranded, double stranded or nucleosomal DNA all to the same extent [44; 50]. In strong contrast the ISWI group of remodelers exhibit ATPase stimulation to the nucleosomal substrates alone and not to DNA substrates [56]. It was also shown that the SWI/SNF group of remodelers have a higher turnover of ATP than that of the ISWI group of remodelers. These remodelers belong to the SF2 family of helicases but lack the double strand displacement activity [44].

1.3.3.4 Nucleosome Distortion/Disruption Processes

The ATP dependant remodelers function by giving rise to a number of events with an end product as a result of distortions and disruptions of the nucleosomal substrate. The DNA that is buried deep inside the nucleosome needs to be exposed so that transcription factors and repair enzymes can nudge their way to such patches of DNA. This is aided by the remodelers. Some of the major nucleosomal disruption processes are summarised below [96] (Figure 13).

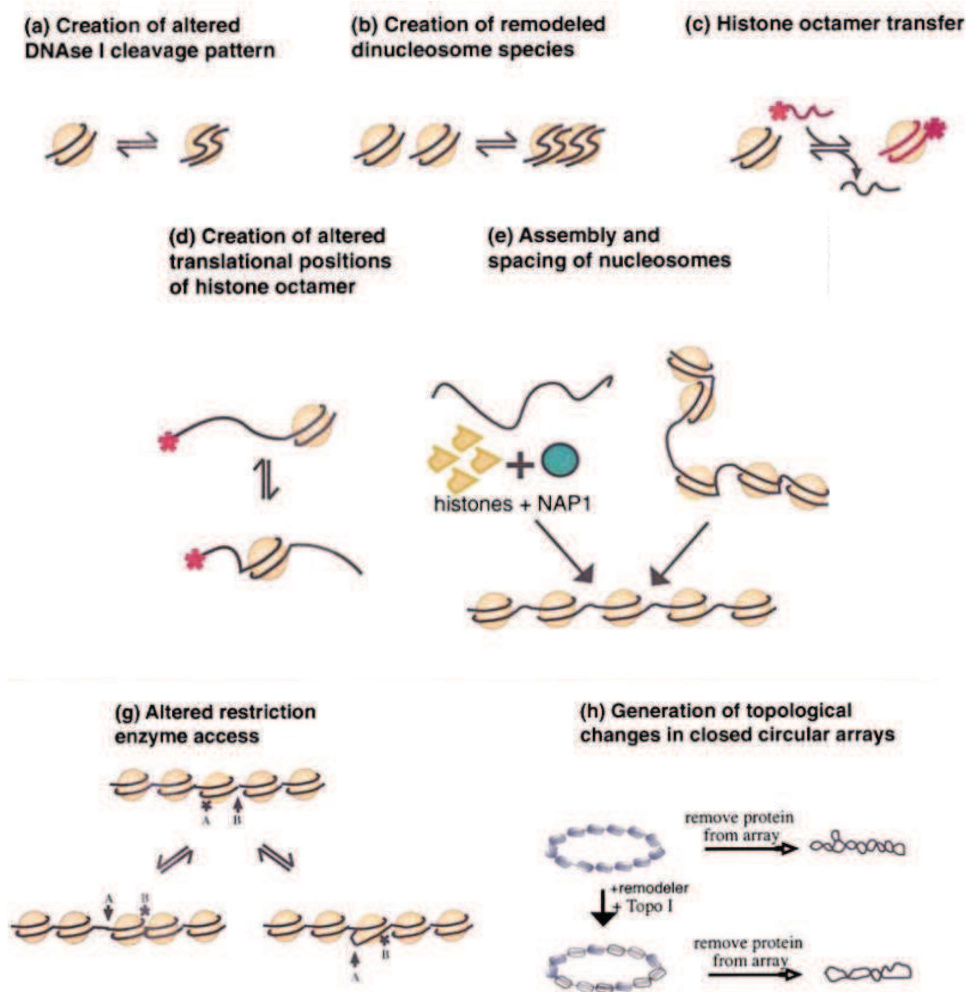

Figure 13: Biochemical properties of ATP dependant Chromatin Remodelers

1.3.3.4.1 Superhelical Torsion

The presence of the helicases in remodelers substantiates the fact that the remodelers behave like helicases. A series of experiments done by Havas and colleagues [97] on different

chromatin remodelers show that the remodelers have a tendency to generate a super helical torsion in DNA. They were able to show that the remodelers like SWI/SNF, BRG1 ISWI and Xenopus Mi-2 were all able to generate super helical torsion in an ATP dependant manner (Figure 13 h).

1.3.3.4.2 Nucleosome sliding

Non ATP dependant mobilization of nucleosomes along DNA or translational positioning can occur probably due to high temperature or ionic conditions [98; 99]. However, the strong interaction between the DNA and the histones makes such an event to occur unfavourable. This is where the ATP dependant remodelers come into play. It has been shown by mobility shift assays that the remodelers are able to mobilize the histone octamer along the DNA [100] (Figure 13 d). The sliding of histone octamer on DNA in an ATP dependant manner was first reported on drosophila extracts [101; 102]. Subsequently, the other remodelers such as NURF, ISWI and CHRAC also exhibited mono-nucleosomal sliding on longer DNA templates [39; 103]. Apart from nucleosome sliding the ISWI group of remodelers were able to generate regularly spaced chromatin templates suggesting their role in chromatin assembly [100] (Figure 13 e). The SWI/SNF group of remodelers on the other hand are able to mobilize the centrally positioned nucleosomes to end positioned nucleosomes (pushing them to the end of the DNA template) away from the thermodynamically favourable position [104]. There is yet another event where the remodeler is capable of shifting the nucleosome 50 bp beyond the DNA template end and such species of nucleosomes are referred to as recessed [105; 106]. While all the above papers show in-vitro evidences of nucleosome sliding, there are also evidences of the same in *in vivo*. The IFN-β promoter when infected with viruses leads to the assembly of the enhancesome and pre-initiation complex. These factors always assemble on a nucleosome free promoter region. When the nucleosome position was analysed before and after the transcriptional activation it was seen that the TATA sequence for TBP binding which was blocked before, moved 35bp downstream allowing TBP to bind [107; 108]. Tsukiyama and group also showed nucleosome sliding in yeast by ISW2. Here, they used a galactose inducible allele of ISW2 to study the structural changes at the level of the promoter [109]. Figure 14 is a pictrographical representation of the different models of nucleosome sliding.

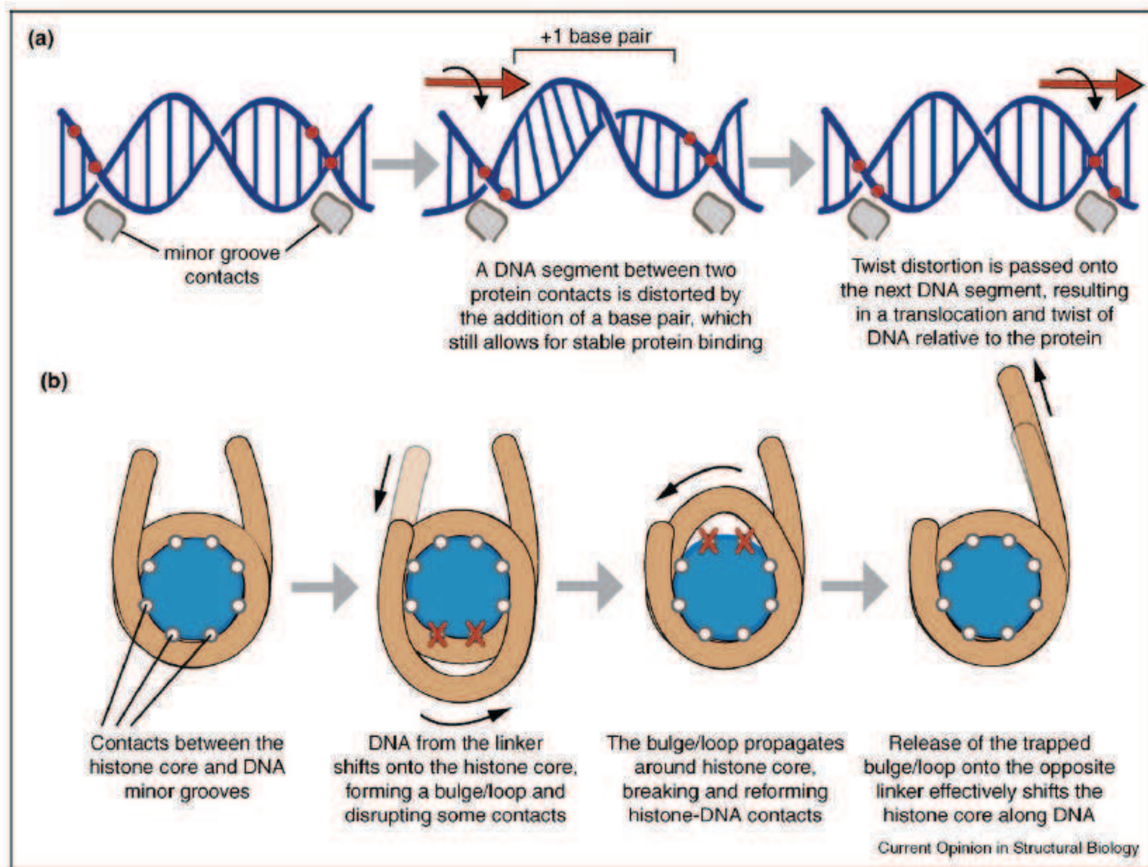

Figure 14: Models for Nucleosome Sliding (a) Twist Diffusion model (b) The Loop/Bulge propagation model. This image is adapted from [110].

1.3.3.4.3 Remodeling of Nucleosomes

When remodelers interact with the nucleosomes in the presence of ATP, it gives rise to structural alterations of the nucleosomes necessary for their mobilization. These remodeler induced alterations are termed remodeling of the nucleosomes (Figure 13 a, b). Nucleosome remodeling can be detected by a number of approaches, including DNase I footprinting, restriction enzyme or nuclease accessibility analysis etc. The experimental data show that SWI/SNF remodelers are able to increase DNase I and restriction enzyme accessibility [96; 111; 112]. Experiments performed on the nucleosome core particle with no free linker DNA were also able to give similar results. In addition hSWI/SNF and ySWI/SNF introduced stable topological changes in closed circular arrays [87; 113]. Site specific cross linking experiments done on the octamer and DNA did not prevent the SWI/SNF remodelers from remodeling the nucleosomes [114].

1.3.3.4.4 Alteration in nucleosome composition

The disruption of the histone octamer with regard to the ATP dependant remodeling complex remains highly controversial. While in the beginning it was suggested that the remodelling by SWI/SNF leads to dissociation of H2A-H2B dimers or alters the histone folds [41; 44], histone crosslinking experiments later showed that the octamer disruption is not necessary for restriction enzyme accessibility [115]. Some studies suggest that dimer expulsion is needed when the nucleosomes are pushed to the end of the template and they are off the DNA template they tend to have a loose dimer-tetramer interaction. Two independent works suggest the transfer of H2A-H2B dimers from a mononucleosomal substrate to H3 –H4 tetramer [116; 117]. The sequences used for this study was the Mouse Mammary Tumour Virus Promoter(MMTV) and these sequences are prone to dimer loses [118]. However, we cannot rule out the *in vivo* evidences that show the exchange or swap of H2A.Z-H2B dimers for H2A-H2B dimers [75; 76; 77].

1.3.3.5 Remosome

Recently our laboratory has made a detailed analysis of the mechanism of action of RSC [111]. High resolution microscopy and novel biochemical techniques were used in this study. Atomic Force Microscopy (AFM) and electron cryomicroscopy show that two types of products are generated during the RSC remodelling reaction of centrally positioned 601 nucleosomes: (i) stable non-mobilized particles, termed remosomes that contained about 180 bp of DNA associated with the histone octamer and, (ii) slid to the end of the nucleosomal DNA particles (Figures 15 and 16). EC-M reveals that individual nucleosomes exhibit a highly irregular trajectory (Figure 15). Restriction accessibility analysis, DNase I footprinting and Exo III mapping clearly show that the histone-DNA interactions within the remosome are very highly altered, particularly at the nucleosomal dyad. The data suggest a two-step mechanism of RSC remodeling consisting of initial generation of a remosome, followed by its mobilization (Figure 16). It was further demonstrated in a series of biochemical experiments that the remosomes are intermediate products generated during the first step of the remodelling reaction that are further efficiently mobilized by RSC. The very recent data of our laboratory shows that, also SWI/SNF, but not ACF, was using the same two step mechanism for nucleosome remodelling (unpublished results).

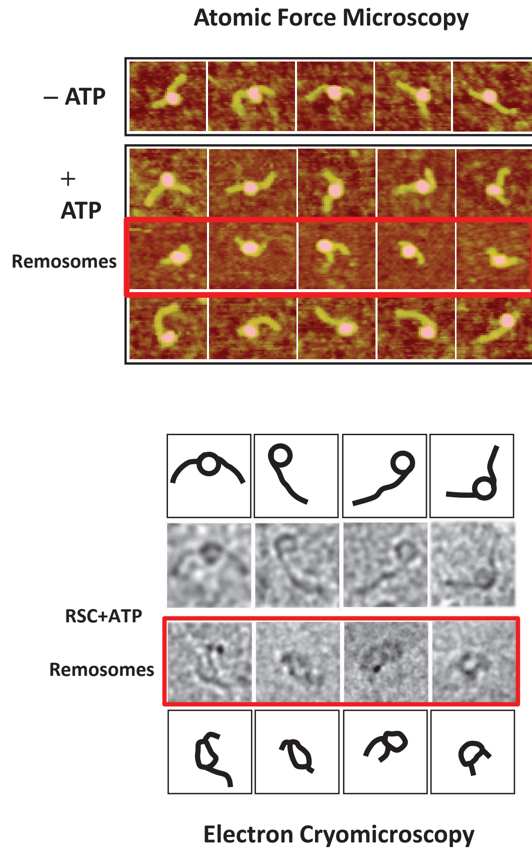

Figure 15: Picture showing the experimental evidences of remosomes.

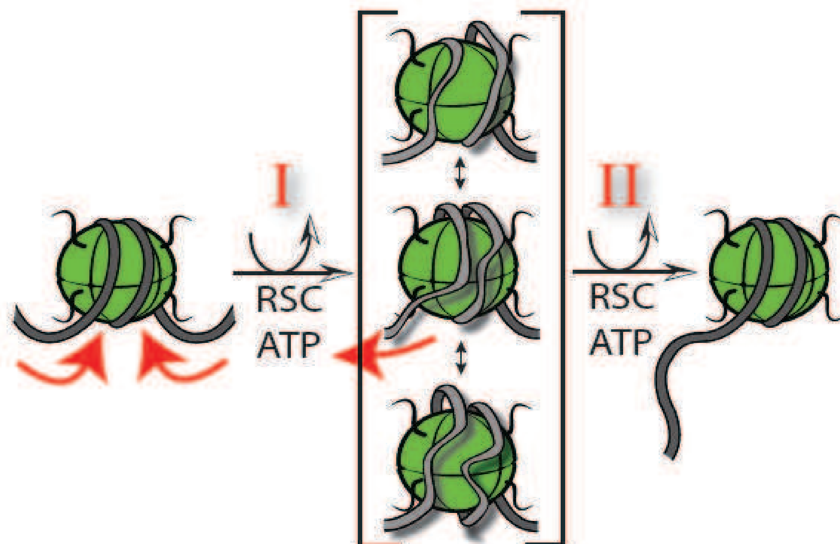

Figure 16: Schematic representation of the two-steps, RSC induced nucleosome mobilization.

1.4 Histone Chaperones

The term “molecular chaperone” was first coined by Ron Laskey [119; 120]. He used the word to describe the nuclear proteins in the extracts of frog oocytes that prevented the incorrect interactions between histones and DNA instead it would end up as aggregates. Lately, the term chaperones has been used to describe the molecules which prevents incorrect interactions. Similarly the histone chaperones protect the interaction between the negatively charged DNA and the positively charged histones.

Histone chaperones are key molecules that play an important role in the assembling and disassembling of the nucleosomes (Figure 17). It has been established that the H3-H4 dimers are assembled into the H3-H4 tetramers on the DNA. This conformation of the H3-H4 tetramer is formed with the help of the histone chaperone and it is this histone chaperone that deposits the tetramer on the DNA. This particular structure is called the “tetrasome” [121; 122]. To this “Tetrasome” two dimers of H2A and H2B are incorporated thus giving rise to the nucleosome core particle. What is important in this step of dimer deposition is that they need to be in the opposite direction and hence are deposited in a step wise fashion. While doing so, there are instances, where the presence of a single H2A-H2B dimer is termed as a “Hexasome”. Histones and DNA do not assemble into nucleosomes under physiological conditions as there is a higher tendency of the histones to no specifically bind to the DNA and leading to aggregates [121; 122]. This is exactly where the histone chaperones come to play their role of aiding in the molecular assembly and disassembly of the nucleosomes. These histone chaperones help in regulating the assembly and disassembly in a less energy consuming fashion.

Histone chaperones are acidic in nature and it is a common trait between most of the chaperones. However they have very little sequence similarity. The histone chaperone Asf1 has a hydrophobic beta sheet on the edge and this mediates with the histones [123]. However, on the other hand the histone chaperone Chz1 has an irregular chain and alpha helices which interacts with the H2A.Z and H2B [124]. Histone chaperones and their functions in particular are very diverse and in order to understand them the chaperones are classified into three categories.

1. Chaperones that bind and transfer histones alone without involving additional partners, for example, Asf1.

2. Chaperones that combine several other histone chaperones subunits, for example, CAF-1.

3. Chaperones that have histone binding capacity and are harboured within large enzymatic complexes, for example Arp-4 in INO80 Chromatin remodelling complex) [125; 126].

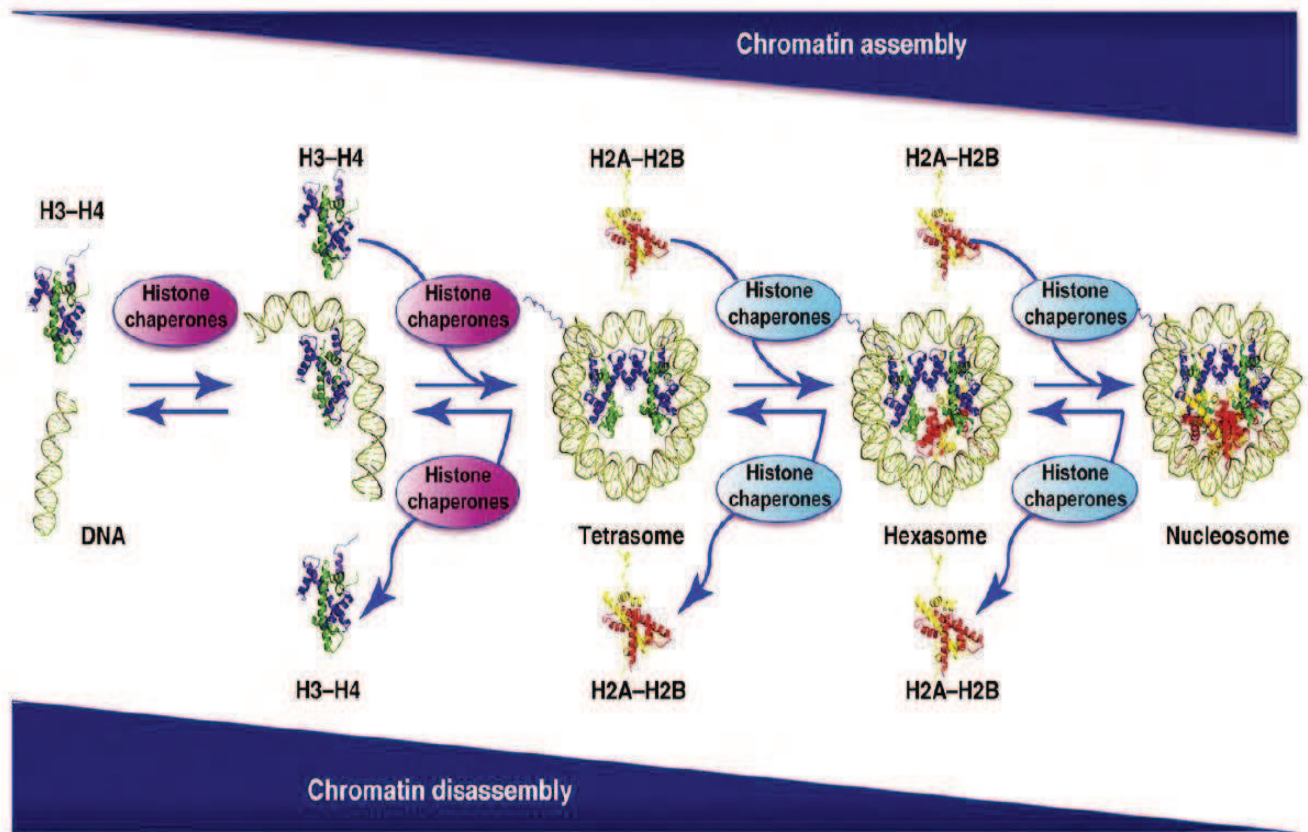

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Figure 17: Histone chaperones aid in step wise assembly and disassembly of the nucleosomes [121].

1.4.1 FACT (Facilitate Chromatin Transcription) complex

Human FACT complex was first identified in the year 1998 as an essential factor for transcription elongation in the chromatin landscape [127]. The heterodimeric complex is composed of two proteins Spt16 and SSRP1. The SSRP1 protein has a homologous counterpart in yeast called Pob3 and a HMG protein called Nhp6. Together they form the yeast FACT. Figure 18 shows the alignment and the domain breakup of the FACT complex in human and in yeast.

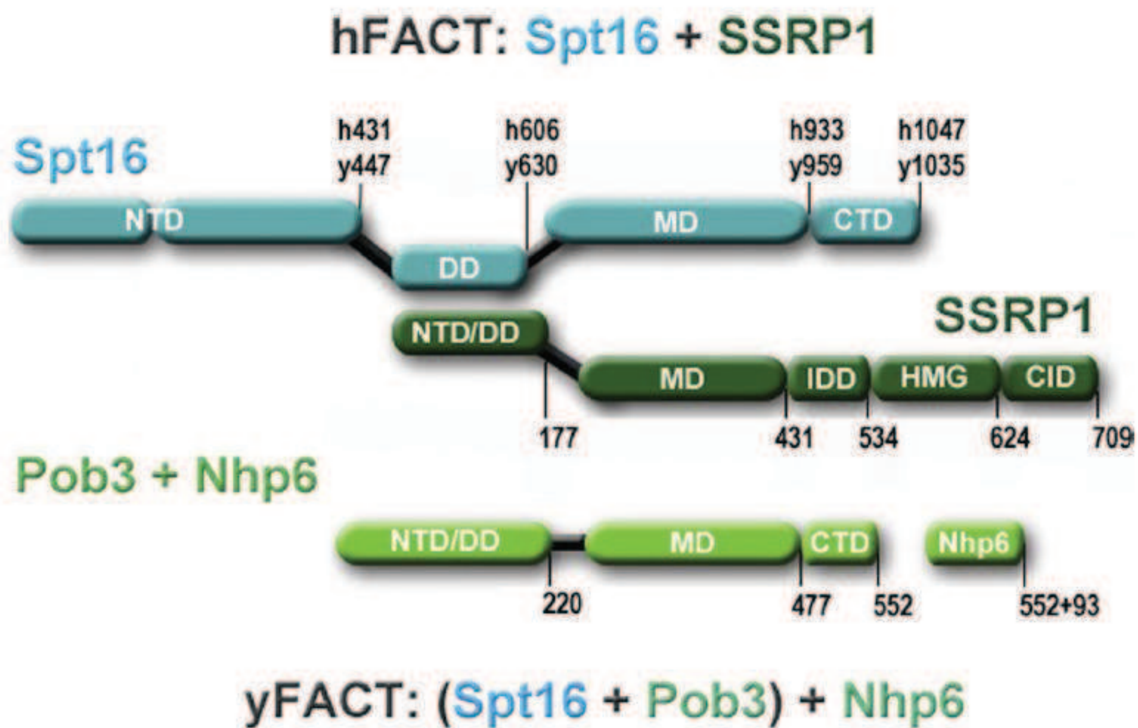

Figure 18: Domain distribution and structural alligment of hFACT againstyFACT. This Image is adapted from a recent review from Winkler and Luger [128].

FACT has been linked to activation of the tumour suppressor protein p53 and to histone variant H2AX-H2B in instances of DNA damage [129; 130]. However, in all three instances be it transcription, replication or repair, FACT functions by reorganising nucleosomes through the disruption of histone-histone and histone-DNA interactions. The FACT heterodimer also has the ability to deposit the H2A–H2B dimer and H3-H4 tetramer on the DNA [131]. It is also worthwhile to mention here that FACT aids in chromatin dynamics during certain critical processes in the cell and also has a histone chaperone activity.

The histone modifications are an important step and are involved wherever the chromatin dynamicity or histone exchange is needed. Modifications of the core histones in the nucleosomes lead to alterations in the structural architecture of the chromatin. This may in turn lead to recruitment of the transcription machinery or a blockage of the machinery. These epigenetic modifications are carried out with the help of factors such as FACT, CHD1, SWI/SNF and ACF [34]. CHD1 a chromatin remodeler is always localised throughout the

coding region of actively transcribing genes with the help of a signal from the trimethylation mark on the Lys-4 of histone H3 (H3K4me3) [81; 82]. CHD1 and FACT physically interact with each other on the nucleosomes in the ORFs of active genes. Similarly, H2BK120ub1 is another epigenetic mark and is associated with the PAF complex and FACT [132]. FACT has also been implicated in repair and is involved in exchange of histone variants such as H2AX. This however is triggered by the phosphorylation of H2AX and this aid in easy exchange of variant histones through FACT [133; 134].

There are also instances where there can be direct modification on the FACT itself. For example poly(ADP-ribosylation) of the Spt16 subunit of FACT by PARP1 (poly(ADP-ribose) polymerase 1 and this type of modified FACT cannot bind on nucleosomes in vitro [135]. Similarly, modification to the SSRP1 subunit of FACT alters the FACT activity levels. There is evidence of direct phosphorylation of the SSRP1 subunit by interaction with CK2 (casein kinase 2) [136]. This phosphorylated subunit of FACT does not bind to the nucleosomes. Instead, when such phosphorylated SSRP1 is accumulated in a storage pool and can lead to sudden activation in vivo. Thus, we can say that the modification on FACT and its subunits moderates the nucleosome organisation and chromatin dynamics.

1.4.1.1 FACT functional models

There are two widely supported mechanisms of how FACT may function. While, on one hand the 'Dimer eviction model' suggests that the histone H2A-H2B dimers are displaced thereby providing access to the nucleosomal DNA [131; 137; 138]. This model comprises of three steps namely FACT binding to nucleosomes followed by FACT mediated H2A-H2B displacement and finally the reinsertion of the H2A-H2B dimers. This window between the displacement and the replacement of the dimers gives an ample amount of time for the RNA polymerase II to perform its function. *In-vitro* experiments suggest that the FACT complex has maximal activity when the FACT to the nucleosome ratio is near 1:1.

On the other hand the 'Global accessibility or non-eviction model' suggests that it is just the reorganisation of the nucleosomes, where the histone DNA contacts are lost, giving a sufficient window for accessibility to factors without histone eviction [139; 140]. This fairly recent model again includes three main steps. The first step involves the binding of Nhp6 which leads to small perturbations and this in turn is an essential step for the recruitment of the ySpt16-Pob3. The second step involves the tethering of the yeast FACT to the histone components in the nucleosome. The final step involves the restoration of the whole nucleosome. A model endorsing the two pathways on FACT is shown in the Figure 19.

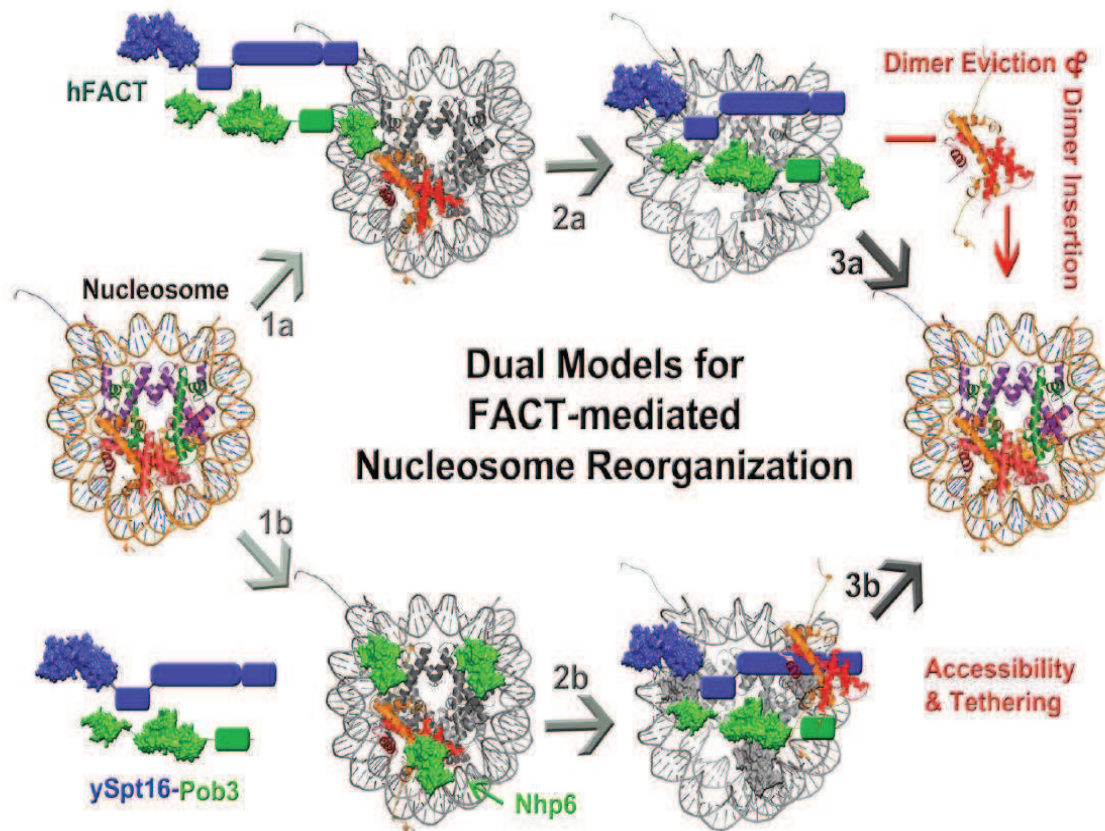

Figure 19: Two Models for FACT mediated Nucleosome Reorganization.

1.5 DNA damage and Repair Systems

1.5.1 DNA Damage

The DNA present in the human body gets damaged due to tremendous endogenous and exogenous factors. The DNA in-spite of taking the beating from these factors bypasses them with a cascade of events where they are capable of repairing and correcting the aberrated DNA with the help of repair enzymes through certain designated pathways. The endogenous factors such as those present in the cytoplasmic environment cause DNA damage by hydrolysis of purine bases, deamination of cytosine to uracil and adenine to hypoxanthine or by oxidation and alkylation [141].The AP-site (Apurinic/Apyrimidiminic Site) is the most frequent and spontaneous type of DNA damage. The next most frequently occurring endogenous damage happens to be that of the deamination of cytosine to Uracil. The other deamination of adenine to hypoxanthine occurs at a lower frequency than that of the former [141].Metabolism also gives rise to certain reactive oxygen species also referred to as ROS that result in oxidation leading to fragmented or open ring structures of the bases or oxidised aromatic derivatives and could give rise to a possible mismatch. S-adenosylmethionine, a metabolic byproduct, is a genotoxic agent that attacks nucleophylic sites on the DNA and generates N-methyl purines and several alkylation lesions [141]. UV and ionising radiations result in the production of radicals, lesions and bulky adducts in the DNA. The above described mutations and damages might lead to genomic instability, cancer and subsequent organism death. All organisms have created very efficient strategies, the DNA repair pathways, to correct these aberrations and to maintain genome integrity and cell viability.

1.5.2 Excision and repair of the DNA damage

The cell has created two major mechanisms for excision of the DNA damage, namely Nucleotide Excision Repair (NER) and Base Excision Repair (BER). NER is a very complicated repair pathway which allows the removal of bulky adducts and photoproducts, attached to the nucleotides. NER functions via a complex, termed nucleotide excision repairosome, which consists of at least 30 proteins. To repair the lesion, the repairosome excises about 30 nucleotides during the repair process [142].

BER is involved in the repair of small alterations of the bases and uses fewer enzymes. Importantly, in human cells 10-20000 DNA lesions are generated per day, which are repaired

by BER [141]. 8-oxoG is the most common base lesion corrected by BER [143]. The glycosylase required for this is OGG1. BER is also implicated in the removal of uracil formed in the DNA by either deamination of cytosine to uracil, thus resulting in U:G mismatch or by misincorporation of dUMP opposite to adenine residues. The glycosylase implicated in the removal of uracil is UDG. We will further describe in more details the mechanism of BER action in both naked DNA and chromatinized templates.

1.5.2.1 Nucleotide Excision Repair

Nucleotide excision repair has an elaborate mode of lesion detection. There are two major pathways in NER: Global Genome NER and Transcription coupled NER (Figure 20). The GG-NER screens first on the basis of disrupted base pairing instead of lesions per se like a patrolling inspector. In Transcription coupled NER, the lesions which prove as a hindrance for the polymerase to carry out transcription are repaired. While the modes of repair in GG-NER and TCR NER are strikingly different in their modes of detection and initial repair, there are overlapping or identical stages in the later part of the repair. There are 25 or more proteins playing a role in NER which are assembled step by step at the site of the lesion and then after the repair is done the complex is disassembled again.

There are three main characterised syndromes which arise due to inborn defects in NER. They are namely Xeroderma pigmentosum (XP), Cockayne Syndrome (CS) and Trichothiodystrophy (TTD). They are all outcomes of extreme sun sensitivity [144]. Xeroderma pigmentosum arises from mutations in any one of the seven genes XPA through XPG and increases the frequency of tumours and induces neurodegeneration. In Cockayne Syndrome, a mutation in the CSA or CSB results in dysmyelination and physical impairment (dwarfism). There is also premature ageing due to increased apoptosis events. With regard to TTD (has some symptoms similar to Cockayne Syndrome) there is brittle hair, brittle nails and scaly skin [145].

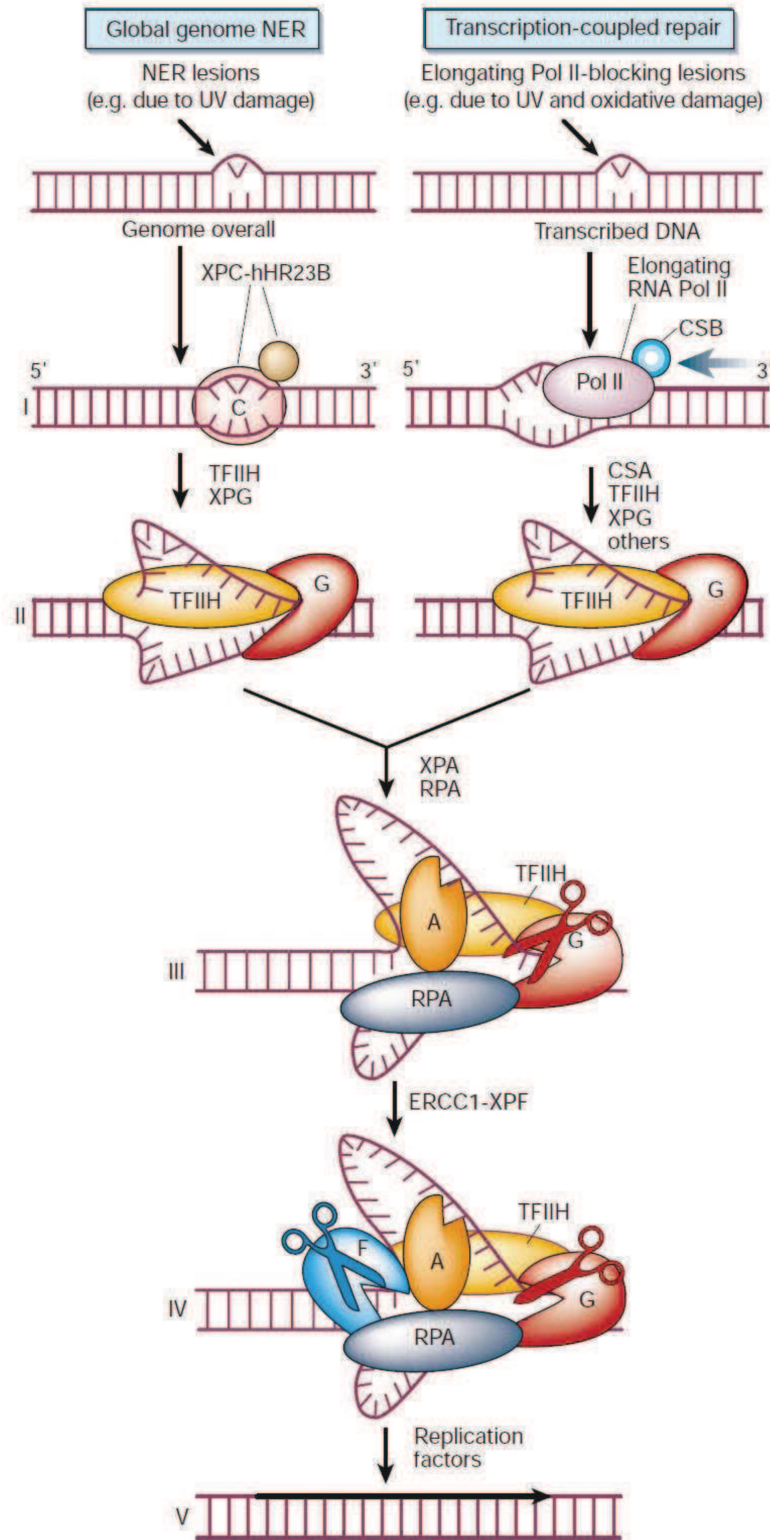

Figure 20: Nucleotide Excision Repair Pathway
This image is adapted from [145].

1.5.2.2 Base Excision Repair

1.5.2.2.1. BER in naked DNA

The different steps of BER on naked DNA templates were well characterized in vivo by using purified recombinant proteins (Figure 21) [146]. BER consists of two main steps. During the first step a specific glycosylase recognizes the modified base and removes it. This results in the generation of apyrimidic/apurinic (AP) site intermediates [147]. This step of the BER pathway is realized in both short patch BER, which involves the removal of only one nucleotide or long patch BER, which is implicated in the replacement of 2-13 nucleotides (Figure 21)[146]. An AP endonuclease activity then cleaved the AP site resulting in the formation of a 3'-hydroxyl and 5'-deoxyribose phosphate or alternatively by an AP lyase producing a 5'-phosphate and a 3'-fragmented deoxyribose. In the short patch BER, the 5'-dRP can be removed by the dRPase activity of polymerase β (pol β) and the 3'-abasic terminus left by AP lyase is cleaved by the 3'-diesterase activity of polymerase β (pol β) and the 3' -abasic terminus left by AP lyase is cleaved by the 3' -diesterase activity of AP endonuclease. The resulting gap is filled by pol β and the remaining nick is sealed by DNA ligase I or III. In the case of long patch BER, however, additional factors such as FEN1, PCNA and RFC are involved. After the formation of the AP site by AP endonuclease, pol γ or pol ε extend the DNA strand from 3'-OH which is accompanied by displacement of the strand containing 5'-dRP for several nucleotides. The resulting flap is removed by the activity of FEN1 producing a nick that is ligated by DNA ligase I. The accessory factors such as PCNA and RFC enhance the activity of DNA pol γ and pol ε in this pathway (Figure 21).

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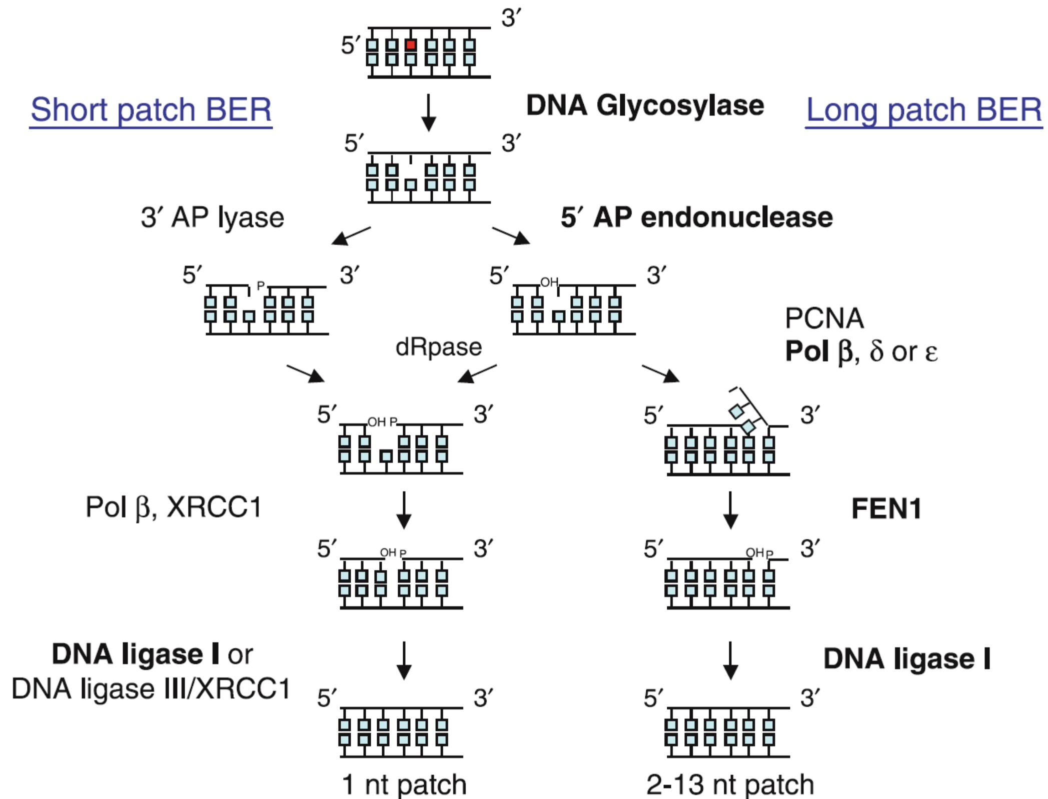


Figure 21: A schematic representation of both short-patch and long-patch base excision repair

1.5.2.2.2 BER in nucleosomes

The nucleosomes, as expected, impede BER. However, the BER enzymes involved in the repair of distinct lesions are differently affected. For example, 8-oxoG was very unefficiently repaired when it was inserted close to the dyad axis of centrally positioned 601 nucleosomes [148]. Indeed, OGG1, APE1 and Polymerase β activities were strongly reduced in such nucleosomes. However, SWI/SNF stimulated the processing of 8-oxoG by each one of the three BER repair factors to efficiencies similar to these for naked DNA. Interestingly, SWI/SNF induced remodelling, but not mobilization of conventional nucleosomes, was required to achieve this effect [148]. Recently, BER was also studied in dinucleosomal templates with or without linker histone H1 [149]. A single 8-oxoG was inserted either in the linker or the core particle DNA within the dinucleosomal template. It was found that in the absence of histone H1 the glycosylase OGG1 removed 8-oxoG from the linker DNA and cleaved DNA with identical efficiency as in the naked DNA. In contrast, the presence of histone H1 resulted in close to 10-fold decrease in the efficiency of 8-oxoG initiation of

repair in linker DNA independently of linker DNA length. The repair of 8-oxoG in DNA within the core particle was very highly impeded in both absence and presence of histone H1. Chaperone-induced uptake of H1 restored the efficiency of the glycosylase induced removal of 8-oxoG from linker DNA, but not from the nucleosomal DNA. However, the removal of histone H1 and nucleosome remodeling are both necessary and sufficient for an efficient removal of 8-oxoG in nucleosomal DNA.

The situation appeared, however, to be quite different for the activity of uracil DNA glycosylase (UDG) which recognises and excises uracil bases from DNA [150]. A recent study used a set of model nucleosome substrates in which single thymidine residues were replaced with uracil at specific locations and a second set of nucleosomes in which uracils were randomly substituted for all thymidines. It was found that UDG efficiently removes uracil from internal locations in the nucleosome where the DNA backbone is oriented away from the surface of the histone octamer, without significant disruption of histone-DNA interactions. However, uracils at sites oriented toward the histone octamer surface were excised at much slower rates, consistent with a mechanism requiring spontaneous DNA unwrapping from the nucleosome. In contrast to the nucleosome core, UDG activity on DNA outside the core DNA region was similar to that of naked DNA. Association of linker histone reduced activity of UDG at selected sites near where the globular domain of H1 is proposed to bind to the nucleosome as well as within the extra-core DNA. These results indicate that some sites within the nucleosome core and the extra-core (linker) DNA regions represent hot spots for repair that could influence critical biological processes.

1.6 Transcription Factor NF-κB (Nuclear Factor Kappa B)

1.6.1 NF-κB

The transcription factor NF-κB plays an important role in many cellular processes and remains a hot area of interest. This transcription factor by the name of NF-κB (Nuclear Factor Kappa B) was first described and reported by Baltimore and Sen as a transcription factor that binds to the enhancer element controlling the formation of the immunoglobin kappa light chain and its expression [151]. It derives its name since it was first reported to be found in B cells. Since then NF-κB has been vividly reported in many journals as a molecule playing an important role in inflammatory responses, immune reactions and in tumorigenesis and cancer. 25 years since its discovery it is one molecule that has been extensively studied and a lot needs to be understood as to how it plays such a crucial role.

1.6.2 The Family Tree of NF-κB proteins

NF-κB is the most widely encountered member of a family of transcription factors, "the dimers of Rel proteins". In mammalian cells, the NF-κB/Rel family contains five members: RelA (p65), c-Rel, Rel B, NF-κB1 (p50 ; p105) and NF-κB2 (p52 ; p100) [152] (Figure 22). These proteins have homology and possess a structurally conserved 300 amino acid sequence called the *REL* region, a structurally similar motif, referred to as the Rel Homology Region (RHR, sometimes also referred as RH). This Rel Homology Region is responsible for locating the binding sequence on the DNA, dimerization with other Rel Proteins and most importantly nuclear localization. Rel proteins are utilized by many eukaryotic organisms and the RH domain remains highly conserved amongst them.

While all Rel proteins share a similar motif, they can still be divided into two classes based upon the sequence at the C-terminal side of the RH. The first class of proteins consists of a long chain of repeats that inhibit their function. The second class of Rel proteins contains a transcription activator region on the C- terminal end of the RH.

Three of the family members, Rel A(p65), c-Rel(Dorsal and Dif in Drosophila), and Rel B, have a transactivation domain(TD) at the C-terminus (Figure 22). The transactivation domains consists of regions rich in serine, acidic and hydrophobic residues which are

essential for trans-activation activity. NF-κB1/p105 and NF-κB2/p100 are the inactive precursors of the p50 and p52 proteins, respectively and in an un-stimulated state; these proteins are localized to the cytoplasm. These proteins are processed proteolytically and their C-terminal domains are removed, thereby providing these proteins admission inside the nucleus [153].

p50 and p52 usually form homodimers or heterodimers with one of the three proteins that has a transactivation domain. RelA and p50 exist in a wide variety of cell types, while c-Rel expression is confined to hematopoietic cells and lymphocytes (Figure 22). The expression of Rel B is limited to highly specific sites, such as the thymus, lymph nodes and Peyer's patches [2]. In addition to the heterodimers p50/p65 homodimers of these Rel proteins also exists. It has been reported that p50 and p52 homodimers do not induce transcription. They are thought to be used as post-induction repressors, following invasion by pathogens [154; 155]. However, it has been demonstrated in vitro that p50/p50 has kB site dependant transcriptional activation [156].

NF-κB is induced by stimuli such as pro-inflammatory cytokines and bacterial toxins such as LPS and exotoxin B and a number of viruses/ viral products (HIV-1,HTLV-1,HBV, EBV, Herpes Simplex) as well as pro apoptic and necrotic stimuli(oxygen free radicals, UV light and gamma irradiation)

Although each NF-κB dimer has a different DNA-binding affinity for kB sites bearing the consensus sequence GGGRNNYYCC (R, purine : Y, pyrimidine : N, any base)[157], their functions often overlap. NF-κB complexes composed solely of family members lacking transactivation domains, such as p50 homodimers, are thought to impose transcriptional repression [158].

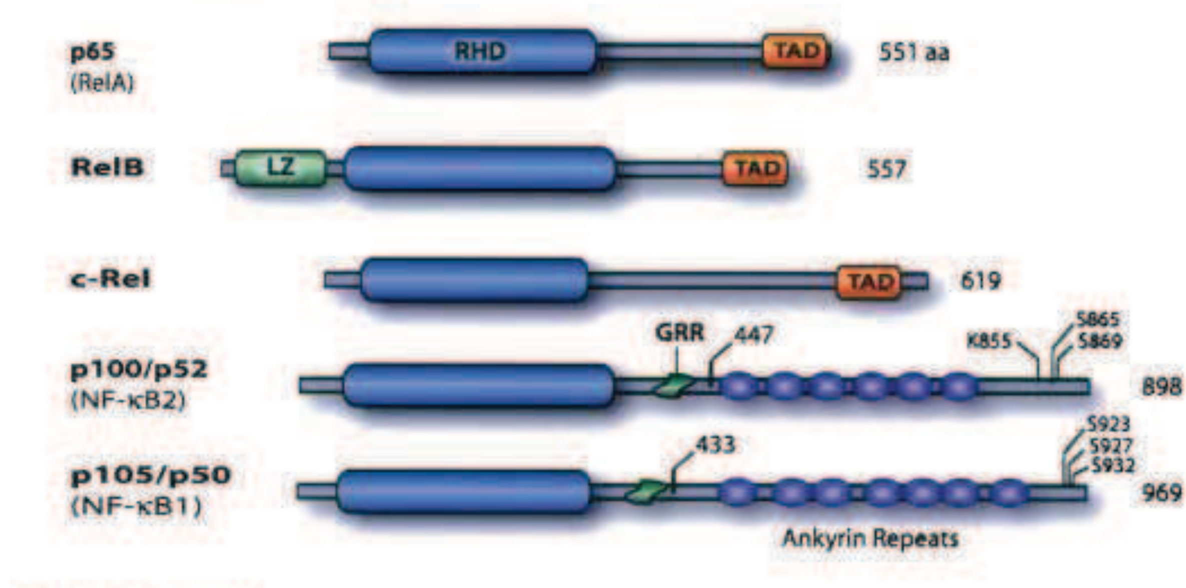


Figure 22: NF-κB family

1.7 UV laser fooprinting

UV irradiation of protein-DNA complexes results in different types of lesions in DNA, the lesion spectrum depending on the presence and type of proteins in these complexes [159; 160; 161; 162; 163] (Figure 23). This dependence is determined by local conformational changes in DNA induced by protein-DNA interactions which can be easily mapped (Figure 25). In fact, UV light "feels" the local DNA structure. Thus, it can be used as a probing agent for the analysis of both protein-DNA interactions and DNA conformation. The method developed for this analysis is called "photofootprinting". The use of UV lasers has many advantages compared to conventional light sources. With a single UV laser pulse a footprint of the protein is achieved. Additionally, high intensity laser irradiation, contrary to conventional light sources, induces specific biphotonic lesions in DNA. These lesions are extremely sensitive to local DNA structure and can be easily detected by treatment with chemical reagents or enzymatic digestion.

Irradiation of DNA with conventional UV light sources produces mainly two types of monophotonic lesions: cyclobutane pyrimidine dimers (CPDs) and pyrimidine (6-4) pyrimidone monoadducts ((6-4) PPs). The efficiency of induction of these photolesions might be affected by the presence of specifically bound proteins. This has been exploited to map *in vivo* the interactions of different transcription factors with their cognate DNA sequence [159; 160; 161; 162]. UV irradiated DNA was digested with T4 endonuclease V (for detection of CPD's) or treated with hot piperidine (for (6-4) PP detection). The sites of the cleavage were found by ligation mediated PCR, followed by separation on a sequencing gel. The above two types of monophotonic lesions can also be induced by using UV laser irradiation, and this was used by Geiselmann and coworkers [163] to study the interactions of integration host factor of E. coli with DNA.

Laser irradiation induces both monophotonic and laser specific oxidative lesions. However, by changing irradiation conditions one can create mostly one type of lesions only. For example, upon high intensity laser irradiation essentially oxidative lesions are formed, the quantum yield of monophotonic lesions being strongly reduced.

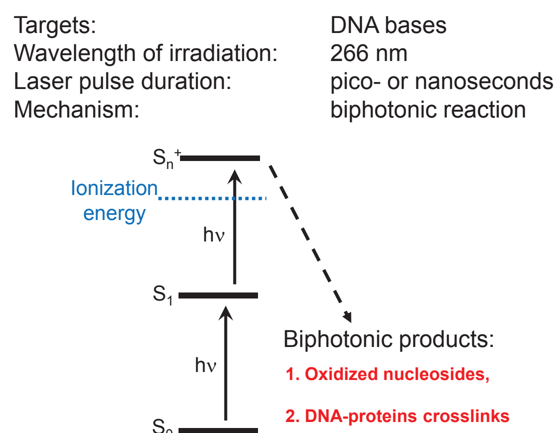


Figure 23: Illustration depicting the biphotonic mechanism of UV laser induced photo lesions.

Two well studied laser-induced oxidative lesions are: 8-oxodG and oxazolone [164; 165] (Figure 24). These lesions are very sensitive to local helical DNA conformation and they can

be analyzed and quantified at the nucleotide level by treatment with specific reagents. 8-oxodG is quantitatively cleaved by Fpg protein, while oxazolone is removed upon hot piperidine treatment [164] (Figure 24). Separation of the cleaved irradiated DNA allows one to find the positions and the relative yield of the lesions [165]. The chemical mechanism of formation of these two types of lesions is well documented. These lesions originate from the competitive transformation process of the transient guanine radical cations precursor generated by a biphotonic absorption and ionization mechanism. The majority stable product 8-oxodG is formed through hydration at position 8 while the less favourable oxazalone is formed upon deprotonation of the radical cation precursor. [165].

Mapping of DNA lesions

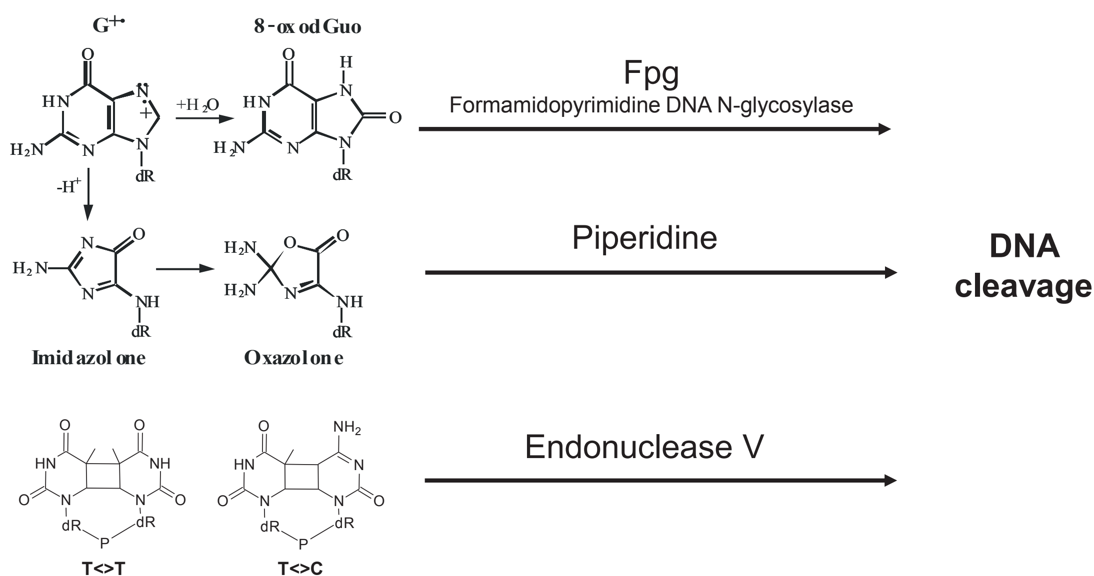


Figure 24: Chemical and enzymatic mapping of DNA lesions generated by UV laser biphotonic ionization chemistry.

We have employed the same technique to study the interaction of the transcription factor NF-κB on nucleosomes. The nucleosomes were constructed on the 601 DNA with the NF-κB binding site introduced inside the sequence at the dyad and at the linker. We performed also similar experiments with the isolated species of nucleosomes, remosomes and slid and compared the transcription factor binding ability.

I.8 Objectives

FACT is an important protein with diverse roles in different vital for the cell processes. Despite the impressive efforts invested how FACT assists, however, DNA repair and transcription remains elusive. The current view is that FACT is facilitating these two processes by modulating the structure of the nucleosomes, but the available data are contradictory and do not allow to have a clear picture of FACT activities.

The two main objectives of the present study are:
(a) To decipher the molecular mechanism of FACT functioning in Base Excision Repair.
(b) To understand how FACT modulates the structure of the nucleosome and gives easier access to the underlying nucleosome DNA sequence by studying the interaction of the transcription factor NF-κB with nucleosomal templates.

We have addressed these questions *in vitro* by using a number of molecular biology and biochemistry methods and reconstituted nucleosomal templates. We discovered that FACT exhibits a “co-remodeling” activity and it is able to boost the remodelling activity of the chromatin remodelers RSC and ACF. Our *in vitro* experiments demonstrate that FACT facilitates both BER and NF-κB binding to nucleosomes via its co-remodeling activity. We anticipate that this property of FACT is essential for its *in vivo* function.

Chapter 2

FACT assists base excision repair by boosting the remodeling activity of RSC

John Lalith Charles Richard[1,2], Manu Shubhdarshan Shukla[1,2], Imtiaz Nisar Lone[2] Khalid Ouararhni[3], Tapas Kundu[4], Ali Hamiche[3], Dimitar Angelov[2], and Stefan Dimitrov[1]

[1]Université Joseph Fourier-Grenoble 1, INSERM Institut Albert Bonniot U823, Site Santé, BP 170, 38042 Grenoble cedex 9, France

[2]Université de Lyon, Laboratoire de Biologie Moléculaire de la Cellule, CNRS UMR 5239, Ecole Normale Supérieure de Lyon, 46 alleée d'Italie, 69364 Lyon Cedex 7, France

[3]Institut de Génétique et de Biologie Moléculaire et Cellulaire, CNRS/INSERM/ULP, Parc d'innovation, 1 rue Laurent Fries, 67404 Ilkirch Cedex, France

[4]Transcription and Disease Laboratory, Molecular Biology and Genetics Unit

Jawaharlal Nehru Centre for Advanced Scientific Research, Jakkur P. O. Bangalore 560064, INDIA

2.1 Summary

FACT protein, in addition to its role in transcription, is involved in the repair of damaged DNA. Although, how FACT assists repair remains elusive. In this work, we have studied the role of FACT in Base Excision Repair (BER). As a model system we have used positioned nucleosomes containing uracil randomly incorporated in nucleosomal DNA. UDG, in agreement with the available data, was able to remove uracils facing the solution, but not uracils facing the histone octamer. FACT alone has no effect on the UDG-mediated removal of uracil. The simultaneous presence of FACT and RSC (a chromatin remodeler involved in repair) allows, however, a very efficient removal of uracil facing the histone octamer by UDG as well as the removal by OGG1 of the otherwise un-accessible oxidative lesion 8-oxoG from nucleosomal templates. This was achieved thanks to the un-expected novel property of FACT to greatly enhance both the remodeling and mobilization activity of RSC. The experimental results reveal that the presence of FACT increases the efficiency of RSC to transform the energy freed by ATP hydrolysis into "mechanical "work. The presented data suggest a stochastic nature of BER functioning *in vivo* with FACT being a key factor in the repair process.

2.2 Introduction

DNA is packaged under the form of chromatin in the eukaryotic nucleus. The nucleosome, the repeating unit of chromatin, consists of an octamer of core histones (two each of H2A, H2B, H3 and H4), around which DNA is wrapped in close to two superhelical turns [166]. The DNA, connecting the consecutive nucleosomes, is called linker DNA and a fifth histone, termed linker histone, interacts with it [166]. Each core histone contains a structured domain, the histone fold, and an unstructured NH2-terminus [167; 168; 169]. Both the linker histone

and the core NH_2-termini are involved in the assembly and maintenance of both the 30 nm chromatin fiber [14; 170] and the mitotic chromosomes [171; 172].

The nucleosome is a barrier for numerous processes requiring access to the naked DNA underlying sequences[23]. The three main strategies that the cell uses to overcome the nucleosome barrier are the posttranslational modifications of core histones[24], the incorporation of histone variants in chromatin[31; 173] and the chromatin remodelers[25; 86]. The chromatin remodelers are very sophisticated nanomachines able to perturb the histone-DNA interactions and to mobilize the histone octamer along DNA by using the energy freed by the hydrolysis of ATP[25; 100; 174; 175]. Depending on the type of ATPase that the chromatin remodelers contain, they are classified in at least four main groups, namely the SWI/SNF, ISWI, CHD and INO80 families [100; 176; 177]. The chromatin remodeler RSC belongs to the SWI/SNF family and it is involved in the repair of damaged DNA [50; 177]. RSC contains a central cavity sufficient for binding of a single nucleosome [92]. We have recently analyzed the mechanism RSC-induced nucleosome mobilization and have shown that RSC generates initially an ensemble of particles with highly altered histone-DNA interactions, which are further mobilized by RSC [111].

Base Excision Repair (BER) is the major pathway that the cell uses to repair lesions induced upon oxidative stress. The different steps of BER functioning on naked DNA are well understood, but how BER functions on chromatinized templates remains elusive. The reported data show that the presence of nucleosomes interferes strongly with BER, although the different BER enzymes are affected in distinct manner[146; 150; 178; 179; 180; 181]. For example, the accessibility in nucleosome DNA of uracil DNA glycosylase (UDG) is reduced down to 30-fold and the removal of histone octamer facing uracil, in contrast to the removal of solution facing uracil, is greatly (up to 3 orders of magnitude) inhibited[150]. No such rotational position dependence of the BER enzyme OGG1 removal of 8-oxoG, the major

oxidative lesion, was, however, observed [148]. Indeed, in this later case the nucleosome remodeling by SWI/SNF was a prerequisite for efficient repair [148; 149].

Human FACT (Facilitates Chromatin Transcription) protein consists of two subunits, hSpt16 and SSRP1, which are both required for functionality [138]. FACT exhibits a histone chaperone activity, it makes a complex with the H2A-H2B dimer and the $(H3\text{-}H4)_2$ tetramer and is able to deposit them on DNA [131]. In addition to its role in transcription, FACT appeared also to be implicated in both replication and repair of damaged DNA [182]. FACT was found in a complex with casein kinase 2 (CK2), which phosphorylates p53 in a DNA damage dependent manner resulting in an increase of p53 activity [129; 136]. FACT was also involved in both phosphorylation and exchange of histone variant H2A.X, two critical events related to the repair of DNA damage [130]. No other data are, however, available on the direct role of FACT in the processes of repair of damaged DNA.

In this work in a series of *in vitro* experiments we have analyzed the role of FACT in BER of uracil containing nucleosomes and we discovered a novel function of FACT in this process. Our data show that FACT greatly facilitates the removal of uracil by UDG from nucleosomal DNA by boosting the activity of the involved in DNA repair chromatin remodeler RSC. This suggests that *in vivo* FACT might act in a concert with RSC to repair damaged DNA.

2.3 Results

2.3.1 Effect of FACT on the efficiency of UDG removal of uracil from nucleosomal DNA

To address the potential role of FACT in BER we have used reconstituted centrally positioned nucleosomes. FACT was purified to homogeneity from HeLa cells by double immuno-affinity procedure (Supplementary Figure 1C and Materials and methods). The histone octamer was assembled with highly purified recombinant core histones (Supplementary Figure 1A) and the nucleosome reconstitution was carried out by using the

601 sequence, containing randomly incorporated uracil [180]. The reconstitution conditions were optimized and essentially all DNA was assembled into nucleosomes (Supplementary figure 1B). The OH and DNAse I footprintings (Figures 2A, 4) showed clear 10 bp cleavage pattern further confirming the proper wrapping of the nucleosomal DNA around the histone octamer in the reconstituted samples. The reconstituted nucleosome samples were then incubated with increasing amount of UDG (Figure 1, lanes 3-6), DNA was isolated and after alkali treatment (to cleave the DNA phosphor-backbone at the abasic site generated upon removal of uracil by UDG) the cleaved DNA was run on a PAGE under denaturing conditions. The cleavage pattern of the free DNA arms of the nucleosome DNA is identical to the cleavage pattern of the naked DNA (Figure 1, bottom of the gel, compare lanes 3-6 with lanes 21-24). Although, the cleavage pattern of the nucleosomal DNA strongly differed from this of naked DNA (upper part of the gel, compare lanes 3-6 with lanes 21-24). Comparison with the OH footprinting of the nucleosome particles (lanes 1 and 25) shows that even at the highest concentration of UDG used, cleavage in nucleosomal DNA is only observed at the sites facing the solution which are also accessible to OH° radicals. This result is in perfect agreement with the reported data

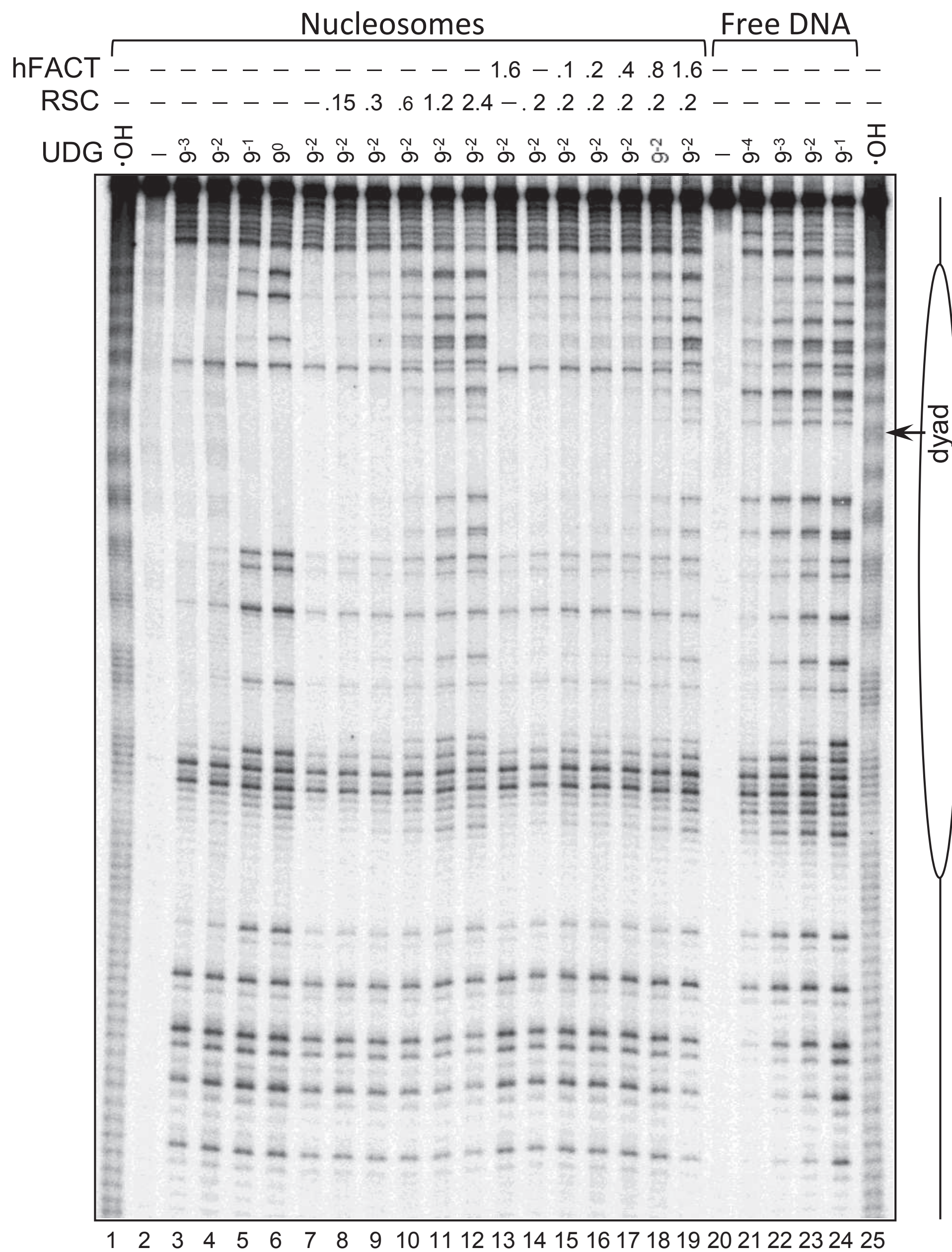


Figure 1. The simultaneous presence of both FACT and RSC, but not FACT alone, is required for efficient UDG excision of uracil at nucleosomal DNA sites oriented towards the histone octamer. Centrally positioned nucleosomes were reconstituted by using ^{32}P 5'-labeled 255 bp 601 DNA fragment containing randomly incorporated uracil residues. (**A**) Lanes 3-6: analysis of the UDG enzymatic activity within the nucleosomal and linker DNA. The nucleosome solution was incubated with the indicated increasing (nine fold step) amount of UDG for 60 minutes at 30°C and the cleavage pattern of the isolated DNA was analyzed using PAGE under denaturing conditions; lane 2, no UDG added lane 1; OH radical footprinting of the nucleosomes. (**B**) Lanes 8-12: RSC induces a highly efficient UDG-mediated excision of uracil at inward facing sites within the nucleosome. Nucleosomes were incubated with increasing (two-fold step) amount of RSC for 50 minutes at 30°C, and after arresting the reaction they were treated with 9^{-2}units of UDG and the isolated cleaved DNA analyzed on denaturing PAGE; lane 8, control with no RSC added in the reaction. (**C**) Lanes 14-19: FACT facilitates the RSC-dependent UDG excision of uracil at inward facing sites

within the nucleosome. Nucleosomes were incubated with increasing (2 fold step) amount of FACT in the presence of 0.2units of RSC and, after arresting the reaction they were treated with 9^{-2}units of UDG. The cleaved purified DNA was analyzed on denaturing PAGE; lane 13, control containing 1.6pmol of FACT with no RSC added. Note that this highest used in the experiment concentration of FACT does not affect the excision of uracil by UDG. (**E**) UDG cleavage pattern of the naked 255 bp 601 fragment. The experiment was carried out as described in (A), but with nine-fold smaller concentration of UDG an each respective point; on the left is shown schematics of the nucleosome.

(REF) and illustrates the inability of UDG to remove uracil from sites facing the histone octamer. Noteworthy, the presence of 1.6 pmol FACT in the reaction mixture does affect neither the efficiency nor the pattern of removal of uracil by UDG (compare lane 13 with lane 4; note that in both cases the same concentration of UDG (9-2units) was used

Pretreatment of the nucleosome samples with increasing amount of RSC changed, however, completely the UDG cleavage pattern of nucleosomal DNA , i.e. it became qualitatively indistinguishable from this of naked DNA (compare lanes 8-12 with lanes 21-24). Thus, the RSC induced remodeling of the nucleosomes renders all uracil residues (including the ones facing the histone octamer) accessible to UDG. Intriguingly, the same effect was observed when a very small amount (0.15 Units) of RSC (this amount of RSC was unable to change the UDG accessibility to uracil, see lane 8) and increasing amount of FACT was used for the pretreatment of nucleosomes (compare lanes 14-19 with lanes 21-24). This shows that *in vitro* FACT and RSC act in concert to facilitate the UDG removal of uracil from nucleosomal DNA and suggests that *in vivo* they could act similarly to assist BER.

2.3.2 FACT boosts both the remodeling activity and the capacity of RSC to mobilize the nucleosomes

To analyze how FACT affects the remodeling activity of RSC we have used DNase I footprinting. Briefly, we have incubated 601 end-positioned nucleosomes with RSC either in the absence or presence of FACT and after arresting the remodeling reaction, the samples were treated in controlled manner with DNase I. The cleaved DNA was then purified and analyzed on a PAGE under denaturing conditions (Figure 2A). The presence of FACT alone, as expected, did not change the clear 10 bp cleavage pattern of nucleosomal DNA (Figure 2A, compare lane 1 with lane 2). This reveals that under the conditions of the experiment, FACT does not alter the structure of the nucleosome. When incubated with 0.2 units of RSC nucleosomes exhibited some alterations in the DNase I cleavage pattern, testifying for some relatively small perturbations in the histone-DNA interactions induced by RSC (compare lane 3 with lanes 1 and 2). Remarkably, the treatment of the nucleosomes with the same amount of RSC, but in the presence of 1.6pmol of FACT, resulted in very pronounced alterations in the DNase I digestion pattern (lane 4). The same altered DNase I digestion pattern was observed with 5-fold more RSC in the absence of FACT (1unit, lane 5). We conclude that FACT exhibits strong "co-remodeling" activity and is able to boost no less that 5-fold the remodeling activity of RSC.

The effect of FACT on the efficiency on nucleosome mobilization by RSC was studied by EMSA (Figure 2B, C). Treatment with 0.2 units of RSC led to mobilization of a very small part of the nucleosomes (not exceeding 20%, see Figure 2B and quantification on Figure 2C). The presence of increasing amount of FACT in the reaction mixture led to a strong increase of the amount of slid nucleosomes and already at the highest concentration (1.6 pmol) of

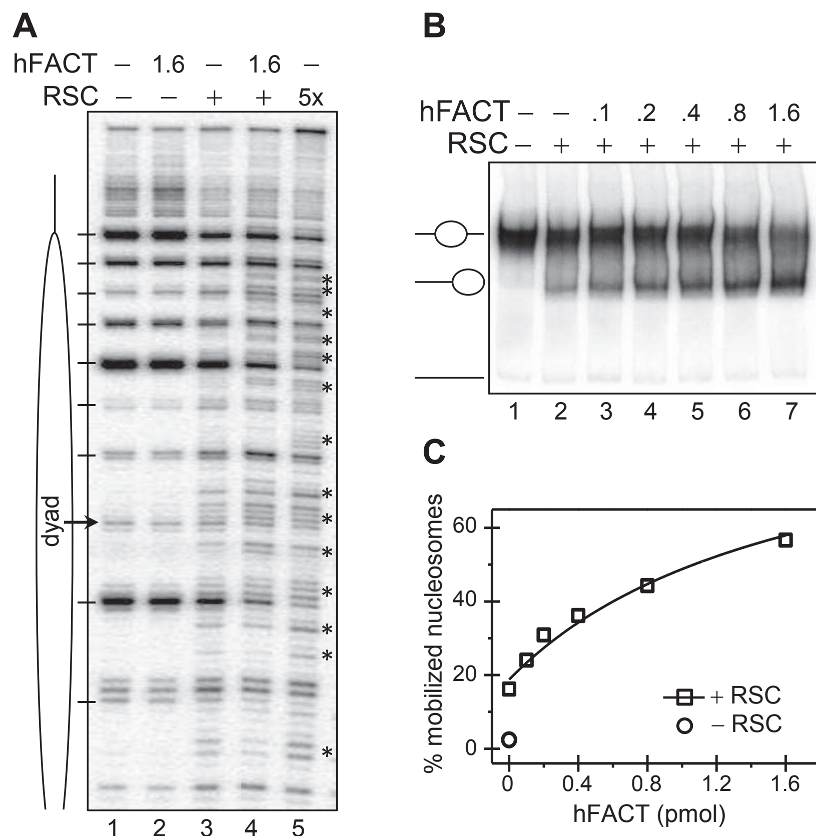


Figure 2. FACT facilitates both RSC-induced remodeling and mobilization of nucleosomes. (**A)** DNase I footprinting. End-positioned nucleosomes, reconstituted on ^{32}P 5'-labeled 241 bp 601 DNA fragment, were incubated with 0.2 units of RSC in the absence (lane 3) or in the presence of 1.6 pmol of FACT (lane 4) for 50 minutes at 30°C; lane 5, same as lane 3, but with 1 unit of RSC; After arresting the remodeling reaction, the samples were digested with 0.1 units of DNase I for 2 minutes, the cleaved DNA was isolated and run on 8% PAGE under denaturing conditions; lanes 1 and 2, controls showing the DNase I cleavage pattern of nucleosomes (lane 1) alone or incubated with 1.6pmol FACT under the conditions described above. (**B**) The presence of FACT increases the efficiency of RSC-induced nucleosome mobilization. Centrally positioned nucleosomes were incubated with 0.2 units of RSC in the presence of increasing amount of FACT, the reaction was arrested and the samples were run on native PAGE. The position of the unslid and slid nucleosomes were indicated; lane 1 control nucleosomes; lane 2, nucleosomes incubated with RSC alone (in the absence of hFACT) (**C**) quantification of the data presented in (B).

FACT, the slid nucleosomes represent 57% from the overall nucleosome population (Figure 2C). Similarly, the presence of FACT in the remodeling mixture led to strong increase in the time-course of the mobilization reaction (Supplementary Figure 2). Therefore, FACT boosts the RSC ability to mobilize the nucleosomes.

2.3.3 FACT efficiently assists RSC to generate nucleosome-like structures exhibiting high accessibility to restriction enzyme

Our recent high-resolution microscopy and biochemical data suggested an intriguing two step-mechanism for RSC nucleosome remodeling (REF). The first step consists in a formation of a stable, non-mobilized particle, termed remosome, which contains ~ 180-190 bp of DNA loosely attached to the histone octamer. The remosome is formed by RSC-pumping of ~ 15-20 bp from each end of the free DNA linkers of the nucleosome without repositioning of the histone octamer. During the second step, the remosome is mobilized by RSC [111].

The main characteristic of the remosome is the higher accessibility of its DNA to restriction enzymes. To test if FACT was able to facilitate the generation of remosomes by RSC we have used the recently developed "in gel one pot assay"[111]. This approach detects quantitatively the alterations in histone-DNA interactions with a 10 bp resolution all along the nucleosomal DNA (Figure 3 and [111; 183]. Briefly, eight mutated ^{32}P-end labeled 255 bp 601.2 sequences were used to reconstitute centrally positioned nucleosomes. A single *Hae*III restriction site (designated as d_0 to d_7, where the subscript refers to the number of helical turns from the nucleosome dyad) was inserted within each of these sequences. The equimolar mixture of the eight reconstituted nucleosomes was incubated with appropriate amount of RSC either alone (to produce ~15% of slid nucleosomes) or in the presence of

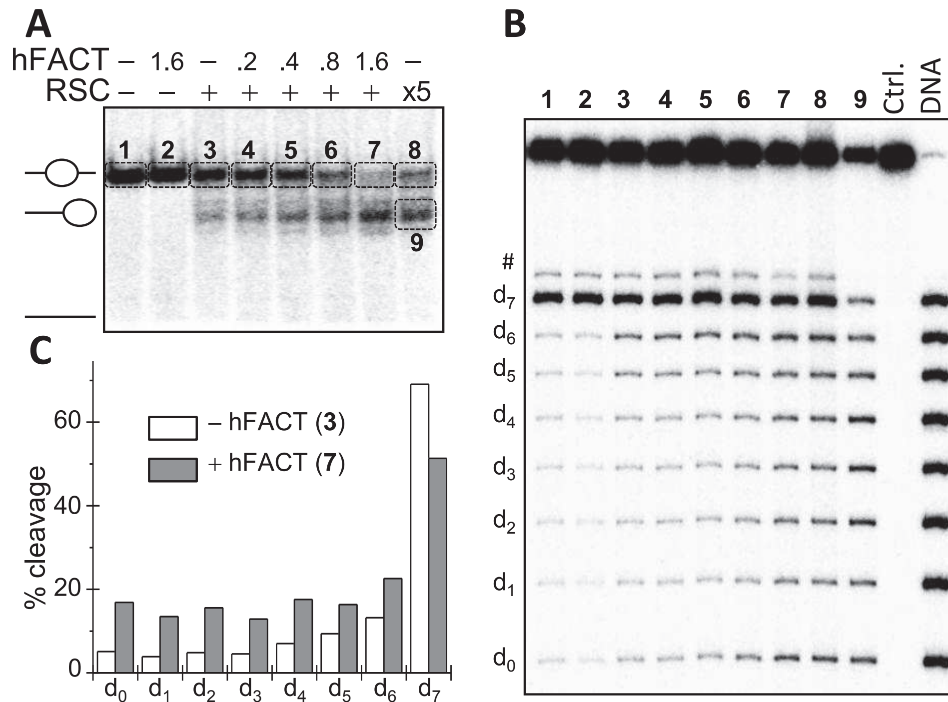


Figure 3. "In gel one pot assay" analysis of the effect of FACT on the DNA accessibility towards *HaeIII* along the length of nucleosomal DNA in control and RSC treated nucleosomes. (**A, B**) Effect of hFACT on RSC-induced remosome generation. (**B**) Preparative PAGE. Centrally positioned nucleosomes were treated with increasing amount of FACT in the presence of 0.2 units of RSC and after arresting the reaction they were separated on native PAGE; last lane, nucleosomes treated with 5-fold higher amount (1 unit) of RSC, in the absence of hFACT; the first three lanes, untreated, and treated with hFACT and with 0.2 units of RSC nucleosomes, respectively. The indicated bands (from 1 to 9) were excised from the gel and in-gel digested with 8 units of *Hae*III for 10 minutes at 30°C. The cleaved DNA was then isolated and separated in 8% PAGE under denaturing conditions (**B**). The positions at the cleavage of the different dyads are indicated on the left; the numbers of each lane refers to the respective excised bands from the preparative PAGE (see A); ctrl, control, non digested DNA; DNA, naked DNA used for reconstitutions of the nucleosomes digested with *Hae*III. (**C**) Quantification of the data presented in (**B**). The # indicates a fragment that corresponds to a *Hae*III site present only in "dyad 7" 601.2 fragment and located at 4 bp from the dyad 7 (d7) site

increasing amount of FACT (Figure 3B). A FACT-concentration dependent mobilization of the nucleosome is observed as judged by the EMSA (Figure 3B). The upper electrophoretic band, containing the remosome fraction as well as the designated slid nucleosome fraction

were excised and in gel digested with *Hae*III. The digested DNA was purified from the gel and run on an 8% PAGE under denaturing conditions. A similar experiment using either control nucleosomes or nucleosomes treated with FACT (in the absence of RSC) was also performed. The gel was then dried and the product bands were visualized by exposure on a PhosphorImager and quantified. As seen (Figure 3C, D), and in agreement with the reported data [111], the accessibility of the control particles (in the absence of FACT) to the restriction enzyme strongly decreases from d_7 to d_0. FACT does not affect this behavior of the *Hae*III digestion pattern. In fact, d_7 and d_6 behaved differently compared to the other positions and showed a high percent of cleavage (up to 50% in the case of d_7). In contrast, the internally located positions (from d_4 to d_0) were poorly cleaved. This is in agreement with the reported data and reflects the mode of association of the histone octamer with the nucleosomal DNA [183]. Noteworthy, these results reveal that FACT alone in the concentrations used does not affect the histone-DNA interactions within the nucleosome.

However, upon incubation with both RSC and FACT, all sites along nucleosomal DNA exhibited highly altered accessibility (Figure 3C, D). The accessibility of d_7 decreased relative to the control particles (this effect is due to the "pumping" of linker DNA in the nucleosome [111], while that of the other positions strongly increased. This increase in the *Hae*III accessibility profile paralleled the increase of FACT concentration used in the remodeling reaction. Since this altered *Hae*III accessibility profile is a remosome specific structural "signature"[111], we conclude that FACT assists RSC in perturbing the histone-DNA interactions in the nucleosome and in the subsequent generation of remosomes.

2.3.4 UDG removes histone octamer faced uracil from both remosomes and slid nucleosomes with the same efficiency

Since FACT increases impressively the capacity of RSC to generate remosomes, its involvement in repair might be mainly associated with this specific to it property. If this was the case, one should expect damaged DNA to be easily repaired within the remosome. We have addressed this question by studying the ability of UDG to remove uracil from remosomes (Figure 4). In agreement with the data in Figure 1, UDG was unable to excise histone octamer facing uracil from the control nucleosome (Figure 4, "nucleosomes"). Although, even at the lowest concentration of UDG (0.003 units) used in the experiments, in addition to solution facing uracil, the histone octamer facing uracil was rather efficiently removed (Figure 4, "remosomes"). Upon increasing the amount of UDG, the efficiency of removal increases and achieves saturation at ~$5x6^{-1}$units of UDG (Figure 4, "remosomes"). Excision of uracil from slid nucleosomes exhibits essentially the same behavior (Figure 4, "slid"). These results reveal that the alterations of the histone-DNA interactions in the remosome are sufficient for achieving efficient repair.

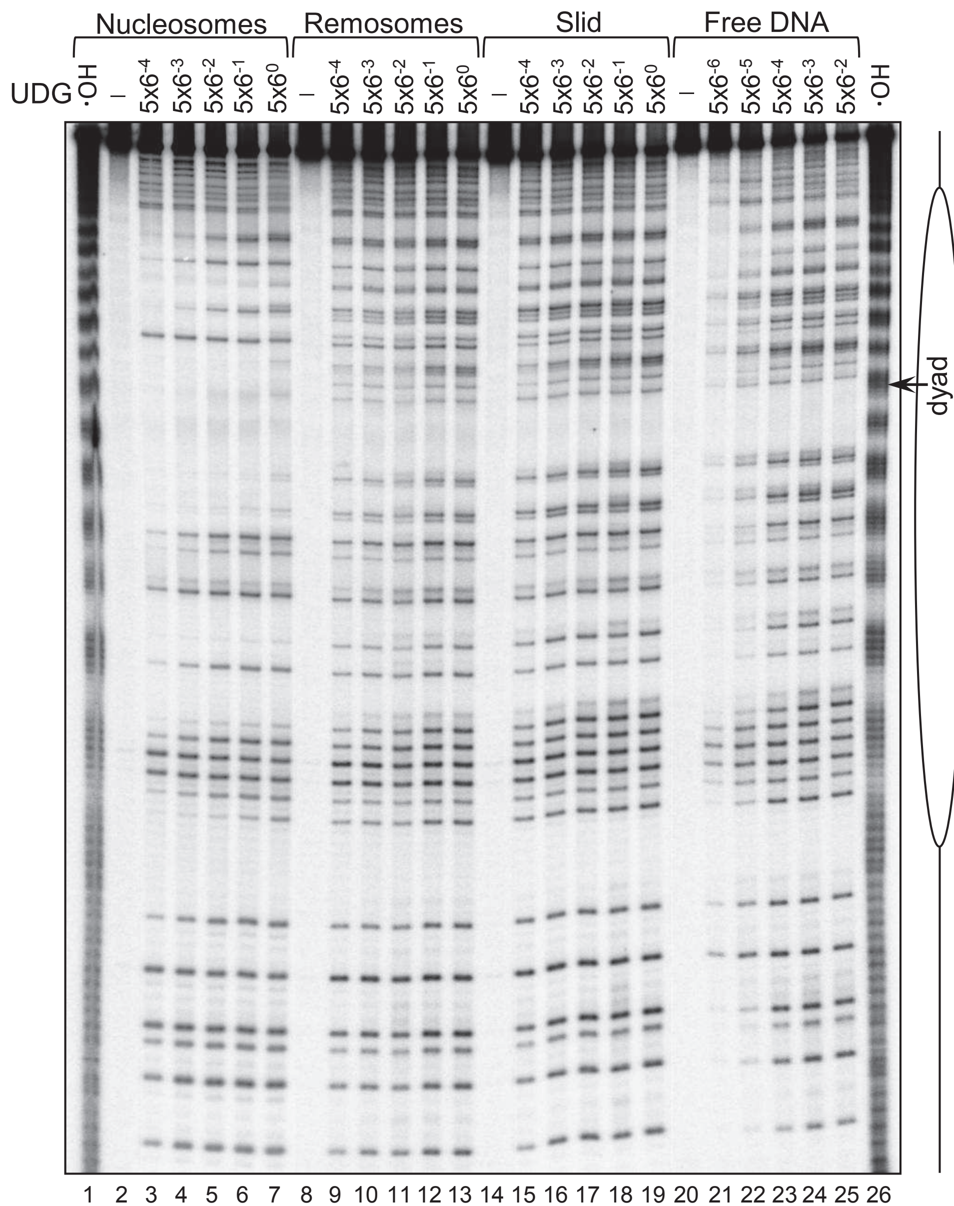


Figure 4. Efficient UDG excision of uracil from RSC-generated remosomes and slid nucleosomes. ^{32}P 5'-labeled 255 bp 601 DNA fragment containing randomly incorporated uracil residues was used for reconstitution of centrally positioned nucleosomes. The nucleosomes were treated with RSC either in the absence of ATP (control particles) or in the presence of ATP to produce ~ 50% mobilized particles. The remodeling reaction was arrested and the samples were separated on native PAGE. The slid nucleosomes and the non-mobilized nucleosomes (containing the remosome fraction) as well as the control nucleosomes were eluted from the gel slice. The particles were then treated with the indicated increasing concentrations of UDG, the cleaved DNA was isolated and run on 8% PAGE under denaturing conditions; DNA, naked 255 bp 601 DNA fragment digested with UDG; first and last lane, OH radical footprinting of native nucleosomes; on the right part of the figure is shown a schematics of the reconstituted nucleosome.

2.3.5 The presence of FACT increases the efficiency of RSC to transform the energy freed by ATP hydrolysis into "mechanical" work

The above presented data reveals that FACT assists very efficiently RSC to both alter the histone-DNA interaction and to mobilize the nucleosome in an ATP-dependent manner. To achieve this, FACT could either act on the nucleosomal substrate or on RSC or on both of them. To differentiate between these possibilities we have carried out the RSC nucleosome mobilization assay at increasing concentration of FACT, but at low ATP concentration (120 μM). At this low concentration of ATP it is possible to precisely measure the amount of ATP hydrolyzed by ATP and thus, to precisely determine the percentage of nucleosomes mobilized by the hydrolysis of a "unit" of ATP. Under the conditions of the experiment, the increase of the FACT concentration results in a 3 fold increase (from 20% to 60 %) of the slid by RSC nucleosomes (Figure 5A). Remarkably, under the same experimental conditions no changes in the amount of hydrolyzed ATP was detected (Figure 5B). This demonstrates that the presence of FACT does not affect the ATPase activity of RSC, while it boosts very strongly the generation of slid nucleosomes, i.e. ~4-5-fold more slid nucleosomes are generated per unit of hydrolyzed ATP in the presence of the highest amount of FACT (1.6pmol) used in the experiments. In other words, the presence of FACT allows RSC to transform much more efficiently the energy freed by the ATP hydrolysis into mechanical work. Since FACT does not affect the ATPase activity of RSC, it should act on the nucleosomes making them more prone to remodeling.

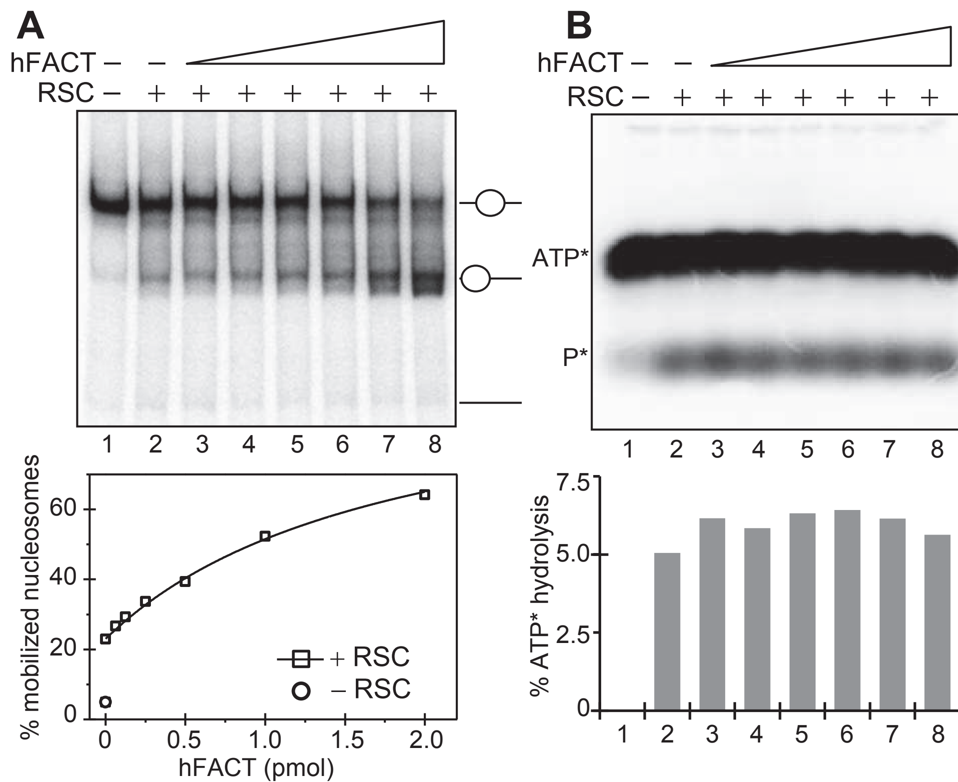


Figure 5. FACT increases the efficiency of the freed by the RSC-mediated hydrolysis of ATP energy used for nucleosome remodeling (**A**) Nucleosome mobilization assay. Centrally positioned nucleosomes were incubated with 0.3 units of RSC at 120 μM of ATP in either the absence or presence of increasing amount of FACT for 50 minutes at 30°C. After arresting the reaction, the samples were run on native PAGE. The bands corresponding to the centrally and end-positioned nucleosomes are indicated. The lower panel represents the respective quantified data (**B**) ATPase hydrolysis assay. Centrally positioned nucleosomes were incubated with RSC (0.3 units) and increasing amount of FACT in the presence of 120 μM of ATP and 3.3μM of $^{32}P[\gamma ATP]$. The products of the ATP hydrolysis were analyzed on 15% PAGE under denaturing conditions. Lower panel shows the respective quantified data.

2.4 Discussion

FACT is involved in several processes including transcription, replication and DNA repair (recently reviewed in[128]. In this work we have studied how FACT assists BER. The presented data reveal that FACT alone has no effect on the removal of uracil by UDG from nucleosomal DNA. Although, FACT exhibits a strong “co-remodeling” activity and it is able to increase many-fold the efficiency of the involved in DNA repair chromatin remodeler RSC to both remodel and mobilize the nucleosomes. FACT does not affect the ATPase activity of RSC, but instead makes the nucleosomes easier to be remodeled and mobilized, i.e. it increases the efficiency of transformation of the energy freed by the RSC-induced hydrolysis of ATP into mechanical work. This allows, in turn, very low amount of RSC to be sufficient to strongly alter the histone-DNA interactions as well as to slide the nucleosomes and thus, the lesions in chromatin DNA to be efficiently repaired. Our data suggest that *in vivo* FACT acts indirect in BER, via RSC, in increasing the efficiency of repair of DNA lesions.

FACT (at the concentrations used in the experiments) does not affect nucleosome structure as judged by both DNase I footprinting and the very sensitive restriction enzyme accessibility assay (one pot assay). In addition, no stable binding of FACT to the nucleosome was detected by EMSA. Then how does FACT act on the nucleosome substrate to make it much easily “remodelable”? It is difficult to answer to this question. Obviously, some transient FACT-nucleosome interactions during the RSC remodeling process should be generated allowing the remodeler to function with much higher efficiency. These transient FACT-nucleosome interactions might require at least some flexibility of the histone octamer, since crosslinking of the histone octamer interferes with the “co-remodeling” activity of FACT (Supplementary Figure S3). Interestingly, FACT assists also very efficiently the nucleosome mobilization by ACF (Supplementary Figure S4), a remodeler belonging to the ISWI family. FACT was also found stably recruited to the CENP-A nucleosomal complexes [184] and RSF, a chromatin

remodeler, is also a member of this complex[185]. In addition, FACT was shown to physically interact with CHD1, another chromatin remodeler [186]. Thus, the cell appears to use the FACT nucleosome reorganization ability to control the activity of distinct remodelers belonging to different families for different purposes in different processes.

Noteworthy, genetic analysis in yeast has revealed evidences for functional relationship between the N-terminal domain of Spt16, one of the FACT subunits, and the docking domain of H2A [187]. The proper folding and integrity of the docking domain of H2A is, however, required for chromatin remodeler dependent nucleosome mobilization [32]. For example, nucleosome reconstituted with deleted docking domain H2A or with the histone variant H2A.Bbd (which possesses defective docking domain) cannot be mobilized by remodelers from both SWI/SNF and ISWI family [32]. This suggests that FACT could, via a transient Spt16-mediated interaction with the H2A docking domain, reorganize the nucleosomes in a way to make them easier remodelable.

As mentioned above, we have not observed structural changes within the nucleosomes due to the presence of FACT alone. Although, data are reported that FACT alone is able to alter the overall nucleosome structure and to destabilize both histone-DNA and histone-histone interactions without any histone eviction [139]. In these last experiments the molar ratio FACT: nucleosome exceeded, however, at least 50 fold the ratio of FACT: nucleosome used in our experiments. (It is worthwhile to mention that the FACT used by the Formosa group consists of at least 50 fold excess of the Nhp6 with respect to the other components of the yeast FACT (Spt16-Pob3) and we are close to equal ratios of the nucleosome and the FACT).

In addition to facilitating UDG removal of uracil, FACT is also able to efficiently assist the OGG1-mediated excision of 8-oxoG (the major oxidative lesion found *in vivo)* from nucleosomal templates in a RSC-dependent manner (Supplementary figure 5). This reveals that FACT can facilitate the function of distinct BER enzymes via specific reorganization of

the nucleosome structure, probably during the required for BER nucleosome remodeling. Importantly, we found that BER acts very efficiently on remosomes and not on non-remodeled nucleosomes. All this suggests that BER could function in a stochastic manner with FACT playing an important role. We propose the following mechanism for BER functioning in vivo (see schematics, Figure 5). The chromatin remodelers from the SWI/SNF family with the help of FACT generate remosomes throughout the genome in a random way. The BER enzymes "scan" genome-wide chromatin and when they detect a lesion within a remosome, they repair it. The remosomes, relatively metastable structures, after being repaired are either spontaneously or with the help of remodelers converted into conventional nucleosomes. This process of remosome "birth and death" is taking place permanently in the cell nucleus and it requires only very small changes in the overall chromatin structure (the remosomes are generated by just pumping 15-20 bp of DNA from both flanking the nucleosome linkers [111]. Such a simple scenario may explain how the 10-20 thousands oxidative lesions generated in a single cell per day are efficiently repaired without the necessity of the BER enzymes to be targeted to the lesions.

2.5 Experimental procedures

2.5.1 Preparation of DNA fragments

1. 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 labelling was performed by using 32P-labeled primer in PCR.

2. 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. Labelling 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.

3. For repair assays the 601 sequence was incorporated with uracil by substituting 0.25% with dUTPs in the normal dNTPs mix comprising dATP, dGTP, dCTP and dTTP with a concentration 25mM.1µl of this new dNTP mix was used in a PCR reaction and random incorporation of uracil is achieved.

4. For end positioned nucleosomes we used the p199.1 plasmid to generate a 250bp fragment with a single linker length of 100bp and the nucleosome at the end.

2.5.2 SDS PAGE electrophoresis of FACT purified from HeLa cell extract

In order to study FACT and it's combined effect with remodelers we characterised the protein FACT on an 18% SDS PAGE. The FACT is purified from HeLa cell extracts and hence this form of hFACT consists of two subunits Spt16 (Suppressor of Ty protein 16) and SSRP1 (Structure Specific Recognition Protein 1). The yeast FACT comprises of two homologous subunits called Spt16 and Pob3. The molecular weight of the 140kDa and 80kDa correspond to the higher molecular weight Spt16 and the lower molecular weight SSRP1. The SDS gel shows the presence of the two subunits of FACT.

2.5.3 SDS PAGE electrophoresis of recombinant histones and octamer

The recombinant core histones were purified to homogeneity and the octamer was reconstituted in equimolar ratios at 2M NaCl. When reconstituted with the DNA fragment of choice the nucleosomes are assembled on the DNA. Here we see the individual hisotnes

separated on an 18% gel and a lane showing the reconstituted octamer and its individual histones in equimolar ratios. (Alongside is a native chicken histone and its histone composition).

2.5.4 Band shift /EMSA of reconstituted centrally positioned nucleosomes

Nucleosome reconstitution was performed by the salt dialysis procedure.

In order to carry out the salt dialysis nucleosome reconstitution 2.5 - 3.0 µg of core histones was mixed with 2.5 µg of carrier DNA and the labelled DNA probe in 2 M NaCl–10 mM Tris–HCl (pH7.4), 1 mM EDTA (pH8.0), 10 mM β- mercaptoethanol, 1mg of Bovine serum albumin per ml in a total volume of 90µl.

The reaction mixtures was thoroughly mixed and transferred to a dialysis tubing and dialysed at 4°C against 10 mM Tris-HCl (pH 8.0)–1 mM EDTA (pH 8.0)–10 mM β-mercaptoethanol containing 1.2, 1.0, 0.8, and 0.6 M NaCl. Each dialysis step was carried out for 2 h. Finally, the reconstituted material was dialyzed overnight against 10 mM Tris-HCl (pH 8.0)–1 mM EDTA (TE) and 10 mM NaCl.

The reconstituted nucleosomes were analyzed on a 5% native polyacrylamide (acrylamide/bisacrylamide, 29:1)–0.25× TBE gel. Under optimal conditions, more than 90–95% of the ^{32}P-labeled fragment was usually nucleosome reconstituted.

In the case of centrally positioned nucleosomes the nucleosomes are assembled on 601 255bp fragment. Here they are able to form a nucleosome with two linker lengths of 52 and 56bp respectively. The gel shift assay/EMSA shows that the nucleosomes are formed and migrate slower compared to free DNA because of its structural retardation in the gel.

2.5.5 DNAse I footprinting of centrally positioned nucleosomes and DNA

Once the reconstituted nucleosome particles are ready we next characterised the nucleosomes by performing a DNase I footprinting. The nucleosomal samples and the DNA fragments were subjected to partial DNase I digestion and then run on a 8% denaturing PAGE - Urea gel. We are able to observe the repeats every 10 base pairs showing the intact DNA and Histone contact points in the case of the nucleosomes and a ladder of bands in the case of the DNA as it is exposed everywhere and is unlike the nucleosomes with the histone octamer protecting the DNA sequence.

2.5.6 OH radical foorprinting of centrally positioned nucleosomes and DNA

The same reconstituted nucleosomes were also subjected to the OH^o radical footprinting. In order to perform the hydroxyl radical footprinting on the mononucleosomes, it was carried out in a 15 µl reaction volume. The hydroxyl radical reaction was carried out by mixing 2.5 µl each of 2 mM $FeAMSO_4$ and 4 mM EDTA, 1M Ascorbate and 0.12 % H_2O_2 in a drop on the side of the tube cap before mixing it with the reaction solution. The reaction was stopped by adding 0.1% SDS, 25 mM EDTA, 1% glycerol and 100 mM Tris pH 7.4. The DNA was purified by phenol chloroform and then run on an 8% denaturing gel. The hydroxyl radical footprinting performed on the 601 255 bp fragment gave rise to a pattern characteristic of the nucleosome. The OH radical performed on the nucleosomes gives rise to a structured repeat of the nucleosomes which are 14 in number and represent the superhelical turns in the nucleosomes, 7 on each side from the dyad. In the case of the DNA we can see a ladder of the sequences showing its overall exposure to the radicals.

2.5.7 Nucleosome remodeling reaction

Typical remodeling reactions were performed with 30 ng of nucleosomes and the appropriate amount 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 mg/ml BSA, 50 mM NaCl, 0.01% NP40) in a volume of 7.5 µl at 29°C for 50 minutes. While RSC is used to slide centrally positioned nucleosomes to end position and ACF is used to slide the end positioned nucleosomes to central positions. The reactions were stopped with 0.1 units of apyrase and 1µg of plasmid DNA pUC18/pUC19.

2.5.8 BER Initiation Assays

The uracil incorporated DNA was formed by PCR and the nucleosomes were reconstituted on this fragment. For carrying out the repair assay the nucleosomes were either subjected to either RSC or FACT or a combination of the two. A fixed concentration of the UDG was used and the samples were incubated in UDG at 29 ºC for an hour. They were then purified by phenol chloroform and precipitated with ethanol and then the reaction was completed using an AP-lyase. We used APE-1 incubated with the DNA to complete the reactions. The fragment was once again phenol chloroformed and ethanol precipitated before running on an 8% denaturing PAGE.

Another method of cleaving the apurinic sites was by treatment with piperidine once the UDG treatment was carried out. In a reaction volume of 9 µl for all the samples 1µl of piperidine was added and heated at 90ºC for 15 minutes. After 15 minutes the tubes were spun at high speed for a short time to bring down the vaporised samples and the heating repeated for another 15 minutes. Once this is done the samples are subjected to phenol chloroform and then ethanol precipitated. The samples were thoroughly suspended in formamide dye heated at 90 degrees for 5 minutes before loading on an 8% denaturing gel.

In the case of BER initiation by hOGG1 the DNA fragment used is 601 DNA but is subjected to UV irradiation before reconstitution. This gives rise to 8-OxoG lesions at places in the DNA fragment. We use human OGG1 to cleave the lesions and then followed by APE-1 digestion to cleave the DNA at the apurinic sites. The DNA fragment is then phenol chloroformed and ethanol precipitated. Then it is thoroughly resuspended in formamide buffer and loaded on an 8% denaturing gel.

2.5.9 In Gel One Pot assay

A sliding reaction was carried out in the presence of increasing concentrations of FACT. Here, the amount of RSC used gave rise to 10% slid and with increase in FACT the nucleosomes are mobilized. Prior to loading on 5% native polyacrylamide gel, 6.25 pmol of not radioactively labelled 255 bp 601 middle positioned nucleosomes were added to each reaction as a carrier in order to maintain stability during subsequent procedures. They are 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).

2.5.10 Gel excision of nucleosomes, remosomes and slid fractions

Centrally positioned 601 nucleosomes were incubated with RSC in the remodeling reaction as described above. Reaction was stopped at 50 minutes by addition of 0.1 units of Apyrase and 1 μg of plasmid DNA, as under these conditions the non-mobilized fraction contains essentially remodelled nucleosome particles. Reaction products were resolved on 5% native polyacrylamide gel. Bands, corresponding to plain nucleosome fraction from 0, un-mobilized fractions from 50 minute, and mobilized fraction from 50 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 cut-off spin filters. Eluted nucleosomes, remosomes and slid particles were divided into equal aliquots, were further subjected to repair by UDG and hOGG1 in the following experiments. Refer to the DNA repair assays for more details.

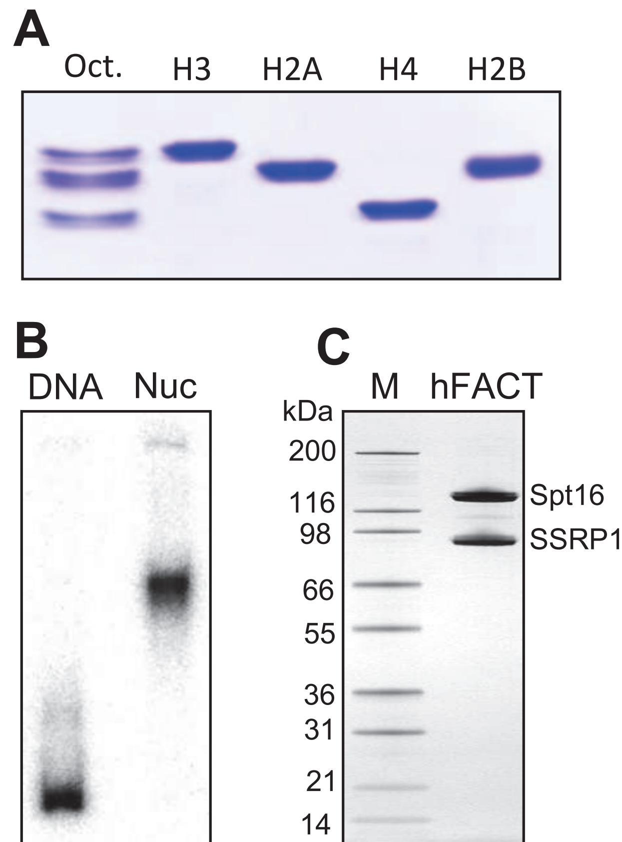
2.5.11 ATPase assays

The ATPase assays were carried out in the remodeling reaction buffer whose composition is mentioned above. However, to slow down the reactions we used a lower concentration of ATP such as 100μM and the ATPase assay was performed on the naked DNA, nucleosomes (centre and end positioned with their respective remodelers). To the reaction mixture 1μl of the source 32P ATP was added. After the remodelling reaction is carried out at 29 degree Celsius for 50 minutes, the reaction was stopped by adding formamide dye and 0.1% SDS. A fraction of the sample was loaded on a 13% denaturing PAGE and migrated at 150 volts for 35 minutes. The gel was dried on a gel drier and exposed.

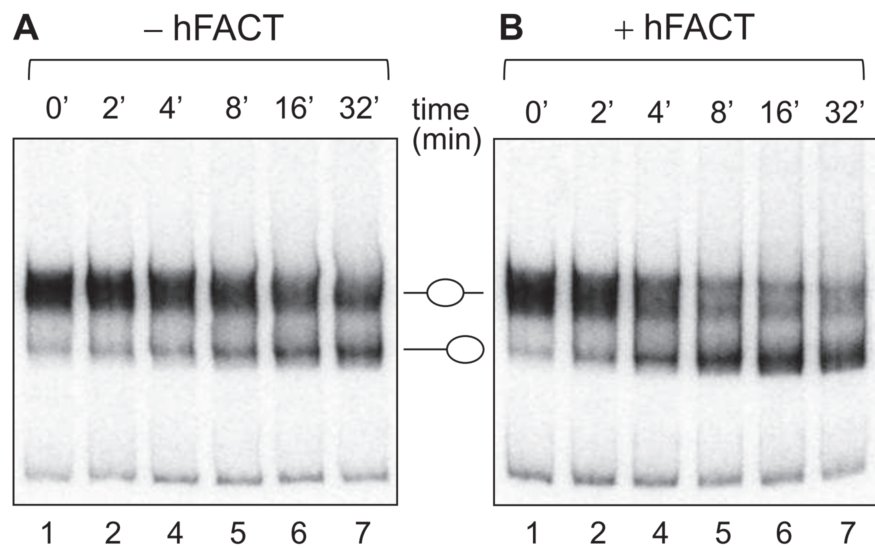
2.5.12 Crosslinking with DMS

DMS is water soluble, imidoester crosslinker. The imidoester functional group is one of the most specific acylating groups available for modification of primary amines. Moreover, the imidoamide reaction product does not alter the overall charge of the protein. The crosslinking of the histones in the nucleosomes were carried out as follows. The nucleosome in itself helps in bringing the histones in the octamer closer to each other for the crosslinking reaction to progess. The free DNA suggests there are no free floating histones in the mixture. DMS is freshly prepared with a concentration of 50mg/ml. The nucleosomes are buffered against 0.3M HEPES pH 10 to remove any remaining traces of Tris. The nucleosome concentration is kept at 50ng/µl to prevent intra nucleosome cross-linking. For a 5 µg nucleosome mix in a reaction volume of 100 µl at pH 10 we add 20 µl of the DMS stock solution. The reaction mixture is incubated at room temperature for 1 hour. The cross linking reaction is stopped by the addition of 2 µl of 1M Tris pH8.0 followed by a buffering the solution against 25 mM Tris for 90 minutes and then against 10 mM Tris overnight. The amount of cross linked histones is checked by running the TCA precipitated histones on a 5-20 % gradient gel.

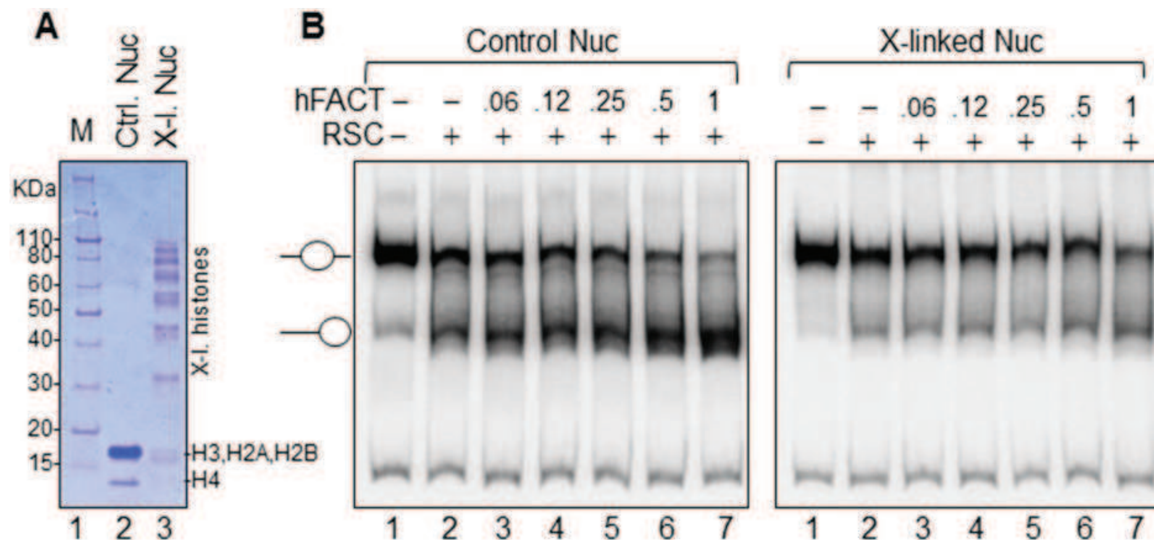
2.5.13 Supplementary Figures



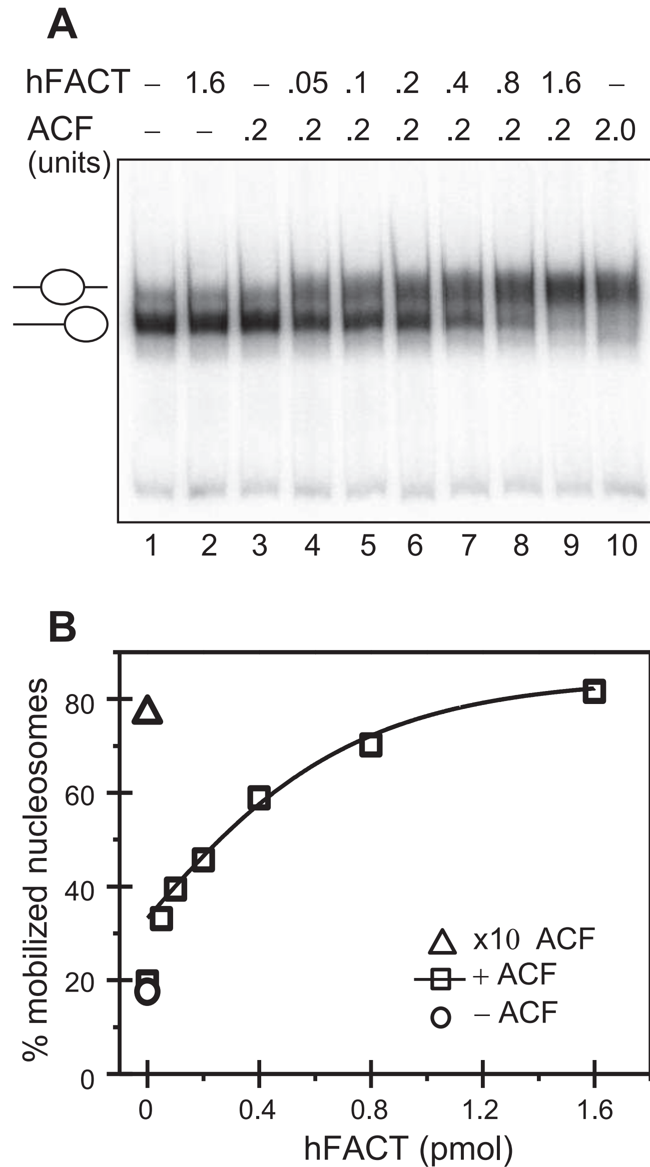
Supplementary Figure 1. Characterization of the reconstitututed nucleosomal particles. (**A)** SDS electrophoresis of purified recombinant histones and the histone octamer. (**B**) EMSA of the 255 bp 601 DNA (left) and reconstituted centrally positioned nucleosomes (right). (**C**) SDS electrophoresis of hFACT . The positions of the two subunits of FACT (Spt16 abd SSRP1 are indicated.



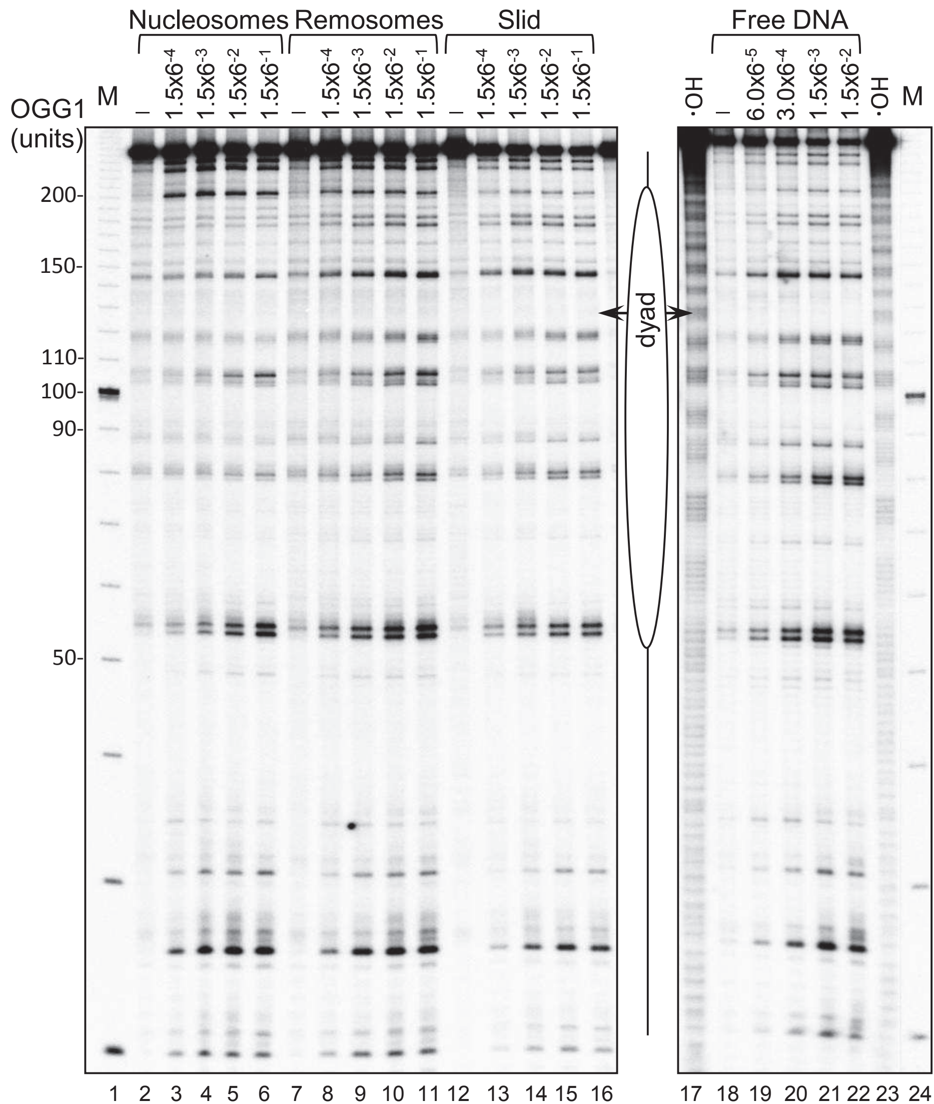
Supplementary Figure 2: Effect of FACT on the time course of RSC-induced nucleosome mobilization. (A) Time course of nucleosome mobilization in the absence (left panel) or in the presence (right panel) of FACT. Centrally positioned 601 nucleosomes were incubated with 0.8 units of RSC and 1.6 pmol of FACT for the times indicated at 30°C in the presence of 1 mM ATP. After arresting the reaction, repositioning of the nucleosomes was analyzed by native EMSA.



Supplementary figure 3: Histone crosslinking affects the co-remodeling activity of FACT. (**A**) SDS electrophoresis of the covalently crosslinked nucleosomes. Centrally positioned 601 nucleosomes were treated with 8mg/ml for one hour and after arresting the histone-histone crosslinking reaction with 180mM Tris, they were run on 4-20% gradient SDS gel; lane 3; lane 2, untreated input nucleosomes. (**B**) Effect of FACT on the RSC-induced mobilization of control nucleosomes (left panel) and crosslinked nucleosomes (right panel). Input or crosslinked see (A). Nucleosomes were incubated with 0.25 units of RSC in the presence of increasing amount of FACT. The reaction products of the nucleosome mobilization reaction were run on native PAGE. The positions of the centrally positioned, slid end-positioned and free DNA are indicated.



Supplementary Figure 4: FACT facilitates ACF nucleosome mobilization. (**A**) EMSA of ACF induced nucleosome mobilization in the presence of increasing amount of FACT. End-positioned 601 nucleosomes were incubated with 0.2 units of ACF either in the absence (lane 3) or in the presence of increasing concentration of FACT (lanes, 4-9). After arresting the reaction, the reaction products were run on a native PAGE; lane 10, EMSA of the nucleosomes incubated with 2 units of ACF in the absence of FACT; lanes 1 and 2, controls showing the input nucleosomes and the incubated with FACT nucleosomes in the absence of ACF, respectively. All reaction solutions contained 1 mM ATP. (**B**) Quantification of the data presented in (**A**).



Supplementary Figure 5: Remosomes are capable of performing the same extent of Base excision repair initiation as that of slid. Centrally positioned nucleosomes were reconstituted on ^{32}P 5' labeled 255bp 601 DNA fragment which is UV irradiated.(A) Analysis of hOGG1 enzymatic activity within the nucleosomal and the linker DNA. The nucleosomal species including nucleosomes, remosomes and slid were gel eluted and then subjected to enzymatic treatment by increasing amounts of hOGG1. The nucleosome species were incubated with hOGG1 at 29ºC for 90 minutes. The DNA was purified before treating with APE1. This enzymatic cleavage of the apurinic sites was carried out at 37ºC for 2 hours. The cleavage pattern of the isolated DNA was analysed using an 8% denaturing PAGE.(B) RSC promotes hOGG1 cleavage of the lesions that are at the edge and are exposed to the solution. Lane numbers 17 and 23 represent a hydroxyl radical footprint of the nucleosomes and helps us determine the parts of the fragment that are facing inside at the octamer and those that are facing the solution.(C) Increasing amounts of hOGG1gives rise to a signature cleavage pattern in the DNA. Lanes 18-22 represent the DNA that is subjected to hOGG1treatment as mentioned in (A).

2.6 Acknowledgements

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Chapter 3:

3.1 Transcription Factor NF-κB and its interaction with the nucleosomes in the presence and absence of FACT

3.1.1 Introduction

As noted in the introduction (Chapter1), FACT is actively involved in transcription [188; 189; 190; 191]. For example, nucleosomes assembled on a high affinity DNA sequence present a polar barrier to transcription of Polymerase II and FACT assists Polymerase II to overcome the nucleosome barrier [192]. In addition, FACT facilitates the polymerase driven transcription by destabilizing nucleosomal structure so that one histone H2A-H2B dimer is removed during enzyme passage [131]. We observed from our previous data that FACT has a co-remodeling activity with chromatin remodelers such as ACF and RSC. FACT acts as efficient fuel conservers and assists the remodeling (generation of remosomes) and mobilization of the nucleosome species with the least energy spent.

With the above in mind, we also wanted to see how the presence of FACT affects the transcription factor interaction with nucleosomes. We hypothesized that FACT, by boosting chromatin remodeling and sliding, might also allow an easier access of transcription factors to nucleosomal templates. If this was really the case, transcription factors should be able to bind to either remodeled nucleosomes (remosomes) and/or slid nucleosomes generated by the concerted action of FACT and RSC. We carried out some biochemical experiments to validate our hypothesis. We decided to use the transcription factor NF-κB in our studies. The transcription factors NF-κB has 150 gene targets but how NF-κB invades the nucleosomal templates remains still elusive.

We have analyzed how the transcription factor NF-κB interacts with the nucleosomes by inserting the NF-κB recognition sequence within nucleosomal DNA. The interaction of NF-κB (p50/p50 dimer) with control nucleosomes, and RSC-generated remosomes and slid nucleosomes was studied by using EMSA, DNase I and UV laser footprinting. The combination of these three techniques allowed us to demonstrate that NF-κB binds specifically only to slid, but not to both control nucleosomes and remosomes.

3.2 Results

3.2.1 Substitution of the NF-κB binding site in the 601 sequence does not alter the nucleosome characteristics.

We have inserted the binding sequence (GGGGATTCCCC) of NF-κB at the nucleosomal dyad (at the center of the nucleosomal DNA, refered here as NF-κB D_0, Figure 1). The nucleosomes were reconstituted by serial salt dialysis. The efficiency of reconstitution was checked by using native PAGE (Figure 2A). As seen, no free DNA was observed in the different samples thus evidencing that under the conditions of reconstitution, all the DNA is associated with the histone octamer. Importantly, both reconstituted nucleosomes exhibited the same DNAase 1 and the hydroxyl radical footprinting patterns (Figure 2B). We concluded that the incorporation of the NF-κB binding sites does affect neither the translational nor the rotational positioning of the 601 nucleosomes. This has allowed us to analyze further with high accuracy the interaction of NF-κB with the reconstituted nucleosomal templates.

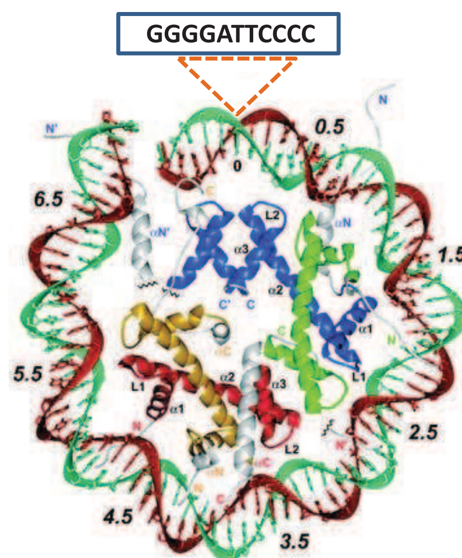

Figure 1: Incorporation of NF-κB binding site in the nucleosome. Schematic showing the wild type 601 sequence and the mutated 601 sequence, containing the NF-κB binding sites at the dyad of the nucleosome (center of the nucleosomal DNA).

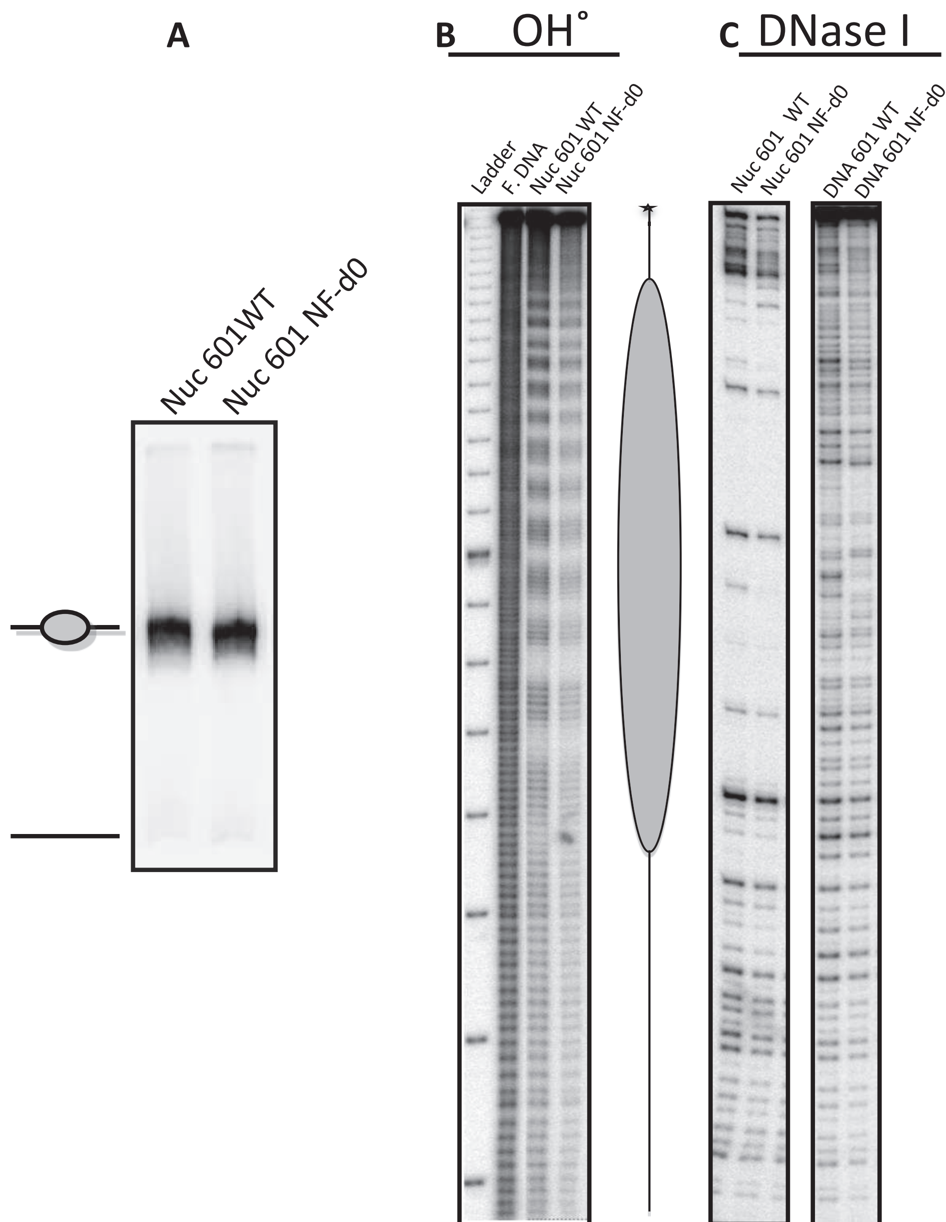

Figure 2: Characterization of the centrally positioned nucleosomes formed on the new templates 601 NF-κB D_0. (A) EMSA on reconstituted nucleosomes using WT- or mutated 601-NF-κB D_0 sequence, which contains the inserted GGGGATTCCCC NF-κB recognition sequence at the nucleosomal dyad. Note that there is no change in pattern or migration of the abovementioned nucleosomes with no free DNA formed. (B) OHº radial footprinting of 601 WT and 601 NF-κB D_0 nucleosomes. (C) DNase 1 footprinting of 601 WT- and 601 NF-κB D_0 nucleosomes. The position of the nucleosome is indicated on the schematics between panel (B) and panel (C).

3.2.2 UV Laser Footprinting demonstrates that the RSC-induced mobilization of nucleosomes, but not their remodeling, is sufficient for NF-κB binding to its cognate DNA sequence

We have analyzed the binding of NF-κB to naked nucleosomal DNA, control (non RSC-treated) nucleosomes, remosomes and slid nucleosomes. The schematic on Figure 3 shows the protocol for isolation of the different species from native PAGE. The species eluted from the gel were allowed to interact with NF-κB and then irradiated with a single 266 laser pulse. DNA was isolated, treated with Fpg protein, and the cleaved samples were resolved on a denaturing gel. The binding of NF-κB to the different probes was also analyzed with EMSA.

As seen, the EMSA shows that NF-κB is able to bind to all of the studied samples (naked DNA, control nucleosomes, remosomes and slid nucleosomes). Indeed, in all four cases higher molecular complexes are formed with much slower electrophoretic mobility (Figure 3). Noteworthy, EMSA does not differentiate between specific and non-specific association of NF-κB with the respective samples. The use of the UV laser footprinting allows, however, to differentiate between specific and non-specific binding (see Chapter 1.7 for more details). Analysis of the UV footprinting data reveals that only naked DNA and slid nucleosomes exhibited the NF-κB UV laser footprinting signature (see Figure 3). No footprints were observed for both control nucleosomes and remosomes (Figure 3). This evidences that NF-κB is able to specifically interact only with naked DNA and slid nucleosomes, i.e. even the perturbed histone-DNA interactions within the remosomes represent a sufficiently strong barrier for NF-κB to interact with its cognate sequences.

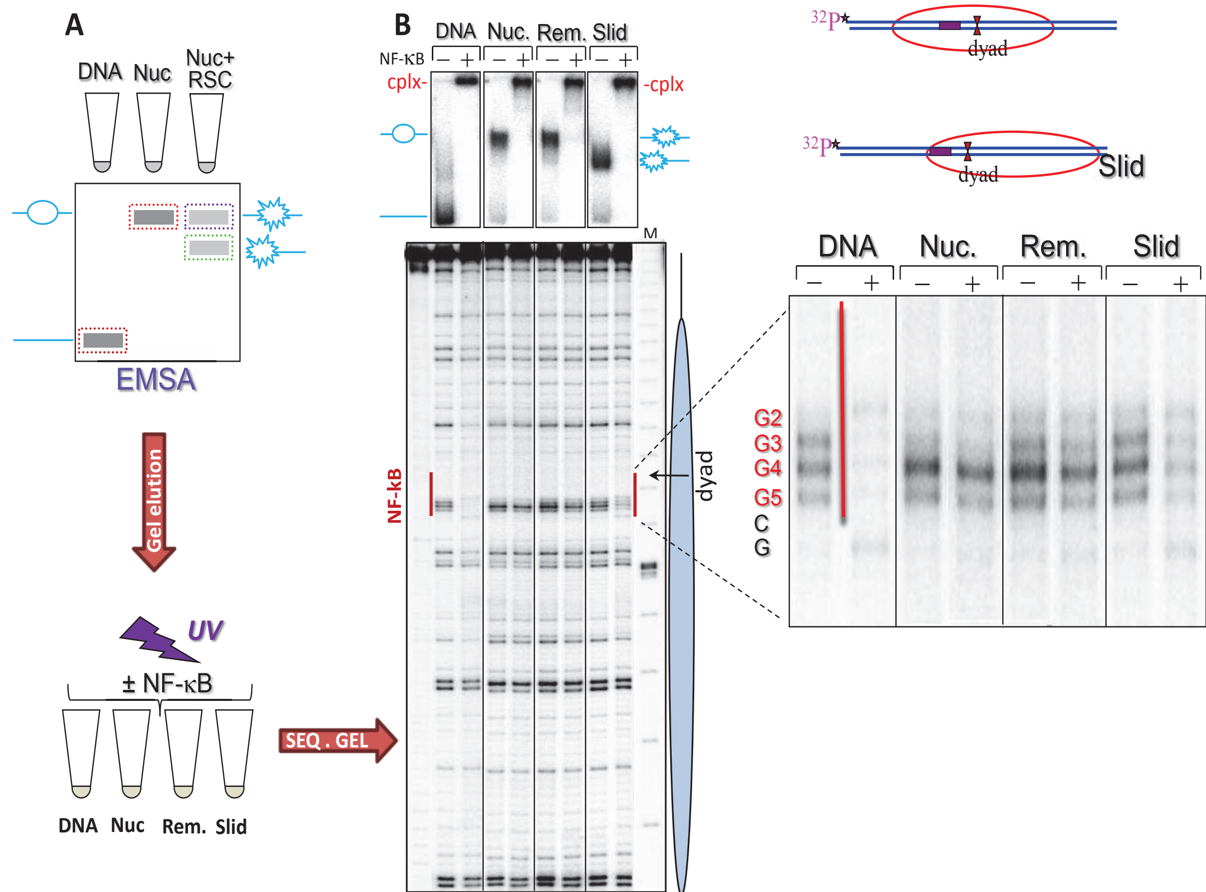

Figure 3: NF-κB binding on the Nucleosome species. (A) Schematics of the isolation of the different nucleosome species. DNA, control nucleosomes and RSC treated mobilized and non mobilized (remosomes) fractions were run on native gel, excised from the gel and after gel elution they were allowed to bind with NF-κB. The NF-κB complexes were then irradiated with the UV laser and treated with Fpg. The cleaved DNA was run on denaturing gel. (B) UV laser footprinting (lower panel) and EMSA (upper panel) of the studied samples. The inset (right) shows an enlarged part of the gel containing the binding sequence of NF-κB. Above the inset, the position of the control and the slid nucleosomes are presented.

3.2.3 FACT boosts RSC mediated mobilization of nucleosomes and aids in the binding of transcription factor NF-κB on the nucleosomes

We hypothesized that FACT would be able to assist the binding of NF-κB to nucleosomal templates through boosting the remodeling activity of RSC. To test this we have incubated 601 NF-κB D0 nucleosomes with either 0.2 u or 1.4 u of RSC (at both these concentrations of RSC a very small part of the nucleosomes was slid) in the presence or absence of FACT. After arresting the mobilization reaction, NF-κB was added to the reaction mixture and allowed to interact with the different templates. Next, the different sample solutions were irradiated with a single UV laser pulse and DNA was isolated and treated with Fpg. The cleaved DNA was then run on a denaturing PAGE (Figure 4). The control naked DNA shows a very clear footprinting reflecting the specific binding of NF-κB to its cognate sequence (Figure 4, compare lane 3 with lane 4). In agreement with the previous data (Figure 3), no binding of NF-κB was observed to the control nucleosomes (Figure 4, lane 6). Importantly, FACT alone was unable, as expected, to help NF-κB to bind to the nucleosome (Figure 4, lane 8). Incubation with 0.2 units of RSC alone or in combination with FACT has again no effect on the capacity of NF-κB to interact specifically with the nucleosomes (Figure 4, lanes 9-12). Although, the concerted action of 1.4 units of RSC and FACT leads to the appearance of a footprinting and thus, to some binding of NF-κB (Figure 4, lanes 13-16).

3.3 Acknowledgements

The research leading to these results has received funding from the European Community's Seventh Framework Programme FP7/2007-2013 under grant agreement number 222008

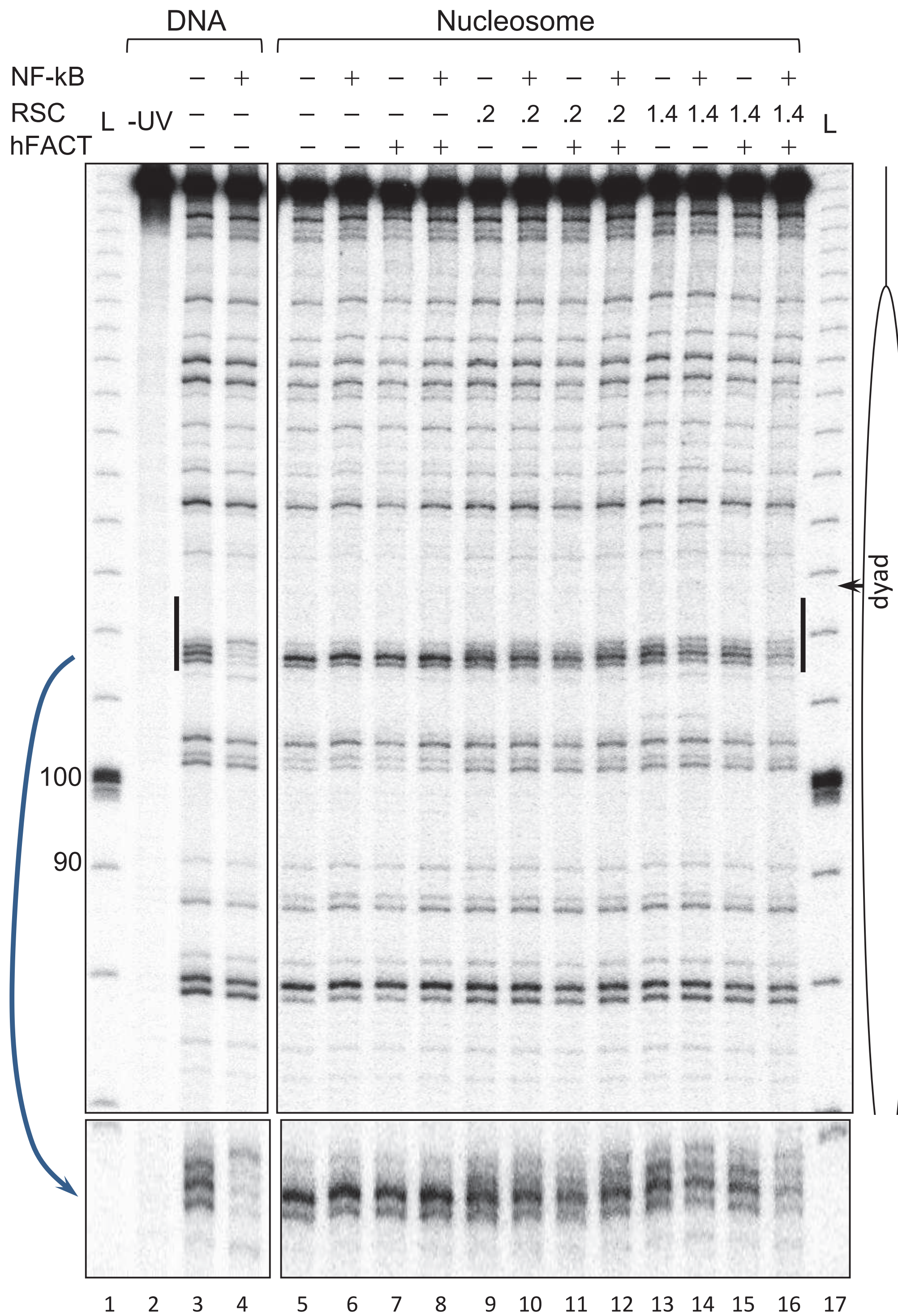

Figure 4: Transcription factor binding in the presence and absence of FACT. Lanes 1 and 17 10 bp DNA ladder. The bottom panel shows the enlarged NF-κB binding sequence. On the right is shown the schematic presentation of the nucleosome..

Chapter 4:

4.1 General conclusions and perspectives

4.1.1 FACT and repair of damaged DNA

FACT is an important molecule that is involved in transcription, replication and DNA repair. We have in this work tried to understand how FACT assists in Base excision repair and transcription. The data presented demonstrates that the FACT by itself is not able to bring about any changes in the structure of the nucleosome but through its co-remodelling activity it is able to increase the efficiency of the remodelers to both remodel and mobilize nucleosomes and thus to facilitates BER. FACT turns out to be a fuel conserver and acts in the efficient conversion of the energy freed by the hydrolysis of ATP by the remodeler in order to have maximum work done. This scenario was shown to be valid for both remodelers RSC and ACF. However, since FACT function is realized by making the remosomes easier "remodelable" we predict that FACT would be also able to boost the activities of chromatin remodelers belonging to other families. This remains to be tested in future.

The presented data show that *in vitro* FACT acts in concert with RSC to assist BER. If this was also happening *in vivo,* one should expect FACT to be associated within the cell with different repair proteins as well as with remodelling factors involved in repair of damaged DNA. We would like to test this hypothesis. Briefly, we plan to establish stable cell lines expressing double tagged (FLAG and HA) SSRP1 (one of the subunits of FACT) and then to purify the FACT complex by using double immuno-purification procedure. The members of the immuno-purified FACT complex will be identified by mass spectrometry. Their identity will be further confirmed by Western blotting using specific antibodies. If we find some remodelling factors involved in DNA repair and/or repair proteins associated with FACT, this will be a strong evidence for the FACT operating mechanism in DNA repair.

The proposed mechanism for FACT functioning in DNA repair predict that FACT should be physically present at the site of DNA damage at least at the beginning of the repair process to allow efficient chromatin remodelling. We will study this by using cell lines expressing GFP-FACT. We expect GFP-FACT to be recruited to the sites of laser-induced damage of DNA. We will follow in real time both the recruitment and the time-course of GFP-FACT

recruitment to damaged DNA by using fluorescence microscopy. If we find that FACT is recruited as predicted to the sites of DNA damage, we will next ask whether the time-course of recruitment of FACT is similar to these of the other identified proteins of the FACT complex (see above). Of particular interest will be to analyse the kinetics of recruitment to damaged DNA of the expected chromatin remodeler(s) associated with FACT *in vivo*.

4.1.2 FACT and NF-κB binding to chromatin

The presented in chapter 3 data suggest that FACT could be involved in the activation of NF-κB dependent genes via boosting the remodelling of the chromatin structure at their promoters. In addition, the available literature data suggest that FACT should assist transcription by allowing an easier Polymerase II passage through the nucleosome. We will study these questions by ChIP using either specific antibodies against FACT or cells which express tagged (HA- and FLAG) versions of FACT (see 4.1.1). In the last case the commercially available high affinity anti-HA and anti-FLAG antibodies would be very helpful. We will analyze both the presence and the time of recruitment of FACT to both the promoters and the coding sequences of early and late NF-κB responsive genes.

Appendix

Wild type 601.2 sequence in pGEM-3Z

CAGTGAATTGTAATACGACTCACTATAGGGCGAATTCGAGCTCGGTACCCTACATGCACA
GGATGTATATATCTGACACGTGCCTGGAGACTAGGGAGTAATCCCCTTGGCGGTTAAAAC
GCGGGGGACAGCGCGTACGTTCGATCAAGCGGATCCAGAGCTTGCTACGACCAATTGAG
CGGCCCCGGGACCAAGCTTCTGCAGGGCGCCCGCGTATAGGGTCCGGGGATCCTCTAGA
GTCGACCTGCAGGCATGCAAGCTTGAGTATTCTATAGTGTCACC

Representation of HaeIII sites in the 601.2 sequences used for 'one pot assay'

CAGTGAATTGTAATACGACTCACTATAGGGCGAATTCGAGCTCGGTACCCTACATGCACA

GGATGTATATATCTGACACGTGCCTGGAGACTAGGGAGTAATCCCCTTGGCGGTTAAAAC

Dyad Dyad1 Dyad2 Dyad3 Dyad4 Dyad5
GGCCGGGACAGGCCGTACGTGGCCTCAAGCGGCCCCAGAGGGCCCTACGAGGCCTTGAG

Dyad6 Dyad7
CGGCCCCGGGAGGCCGCTTCTGGCCGGCGCCGGCCTATAGGGTCCGGGGATCCTCTAGA

GTCGACCTGCAGGCATGCAAGCTTGAGTATTCTATAGTGTCACC

Primers for 282 bp fragment :

New_Trav_link_2nd: 5' CAGTGAATTGTAATACGACTC AC 3'
AT_Rev223: 5' GGTGACACTATAGAATACTCAAGC 3'

Primers for 223 bp fragment :

AT_For: CAGGATGTATATATCTGACAC
AT_Rev223: GGTGACACTATAGAATACTCAAGC

601WT (pGEM3Z-601):

CTATCCGACTGGCACCGGCAAGGTCGCTGTTCAATACATGCACAGGATGTATATATCTGA
CACGTGCCTGGAGACTAGGGAGTAATCCCCTTGGCGGTTAAAACGCGGGGGACAGCGCG
TACGTG**C**GTTTAAGCGGTGCTAGAGCTTGCTACGACCAATTGAGCGGCCTCGGCACCGGG
ATTCTCCAGGGCGGCCGCGTATAGGGTCCATCACATAAGGGATGAACTCGGTGTGAAGA
ATCATGC

Primers for 255 bp fragment:

601-Eco: GCTCGGAATTCTATCCGACTGGCACCGGCAAG
601-Bst: GCATGATTCTTAAGACCGAGTTCATCCCTTATGTG

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See :

The docking domain of histone H2A is required for H1 binding and RSC-mediated nucleosome remodeling

Manu Shubhdarshan Shukla1,2, Sajad Hussain Syed1,2, Damien Goutte-Gattat1, John Lalith Charles Richard1,2, Fabien Montel3, Ali Hamiche4, Andrew Travers5,6, Cendrine Faivre-Moskalenko3, Jan Bednar7,8,9, Jeffrey J. Hayes10, Dimitar Angelov2,* and Stefan Dimitrov1,*

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SI Text

Figure Legends

Figure S1. Expression and purification of H2A COOH-terminal deletion and chimeric proteins and hydroxyl radical footprinting of nucleosmes. **(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 punctuated line below the sequence. **(B)** 18% SDS PAGE of different histones and H2A COOH-truncated mutant proteins. All the proteins were bacterially expressed in denaturing condition and purified from inclusion bodies using SP-sepharose medium. **(C)** Characterization of conventional, variant and mutant nucleosomes by •OH footprinting**.** The gel shows •OH radical cleavage profile of the indicated nucleosomes reconstituted on 205 bp 3'-labeled 601 DNA fragment. Note the higher background (smaller contrast) observed in the cleavage pattern of H2A.Bbd, ddBbd and d79 nucleosomes.

Figure S2. One pot restriction accessibility assay of conventional and H2A.Bbd nucleosomes. Both types of nucleosomes were digested with *Hae* III and samples were processed and percentage of *Hae* III cleavage was quantified as described for Figure 2. Cleavage efficiency of conventional (triangles) and H2A.Bbd (Squares) nucleosomes are presented.

Figure S3. Scans of hydroxyl radical cleavage profile (See Figure 4B) for H2A, H2A.Bbd and H2A.ddBbd containing dinucleosomes in absence or presence of linker histone H1**.**

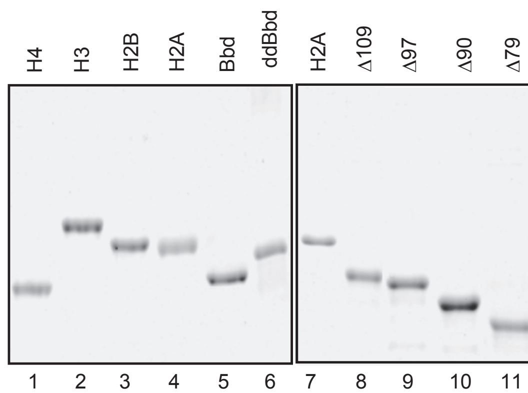
A

```
hH2A.1   M----SGRGKQGGKARAKAKTRSSRAGLQFPVGRVHRLLRK
hH2A.Bbd MPRRRRRRGSSGAGGRGRTCSRTVRAELSFSVSQVERSLRE
```

```
hH2A.1   GNYSERVGAGAPVYLAAVLEYLTAEILELAGNAARDNKKTRIIP
hH2A.Bbd GHYAQRLSRTAPVYLAAVIEYLTAKVLELAGNEAQNSGERNITP
```

```
hH2A.1   RHLQLAIRNDEELNKLLGRVTIAQGGVLPNIQAVLLPKKTESHHKAKGK
hH2A.Bbd LLLDMVVHNDRLLSTLFNTTTISQ--VAPGED----------------
```

B



C

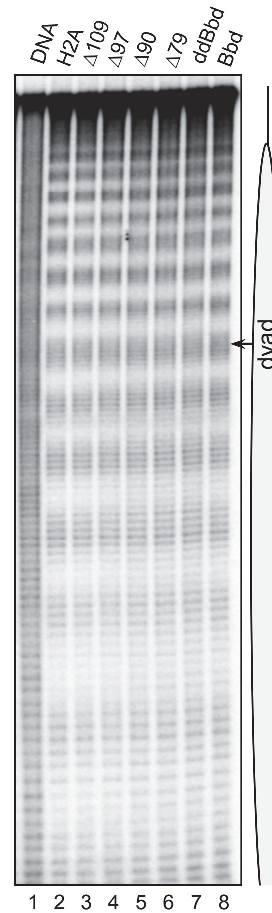


Figure S1

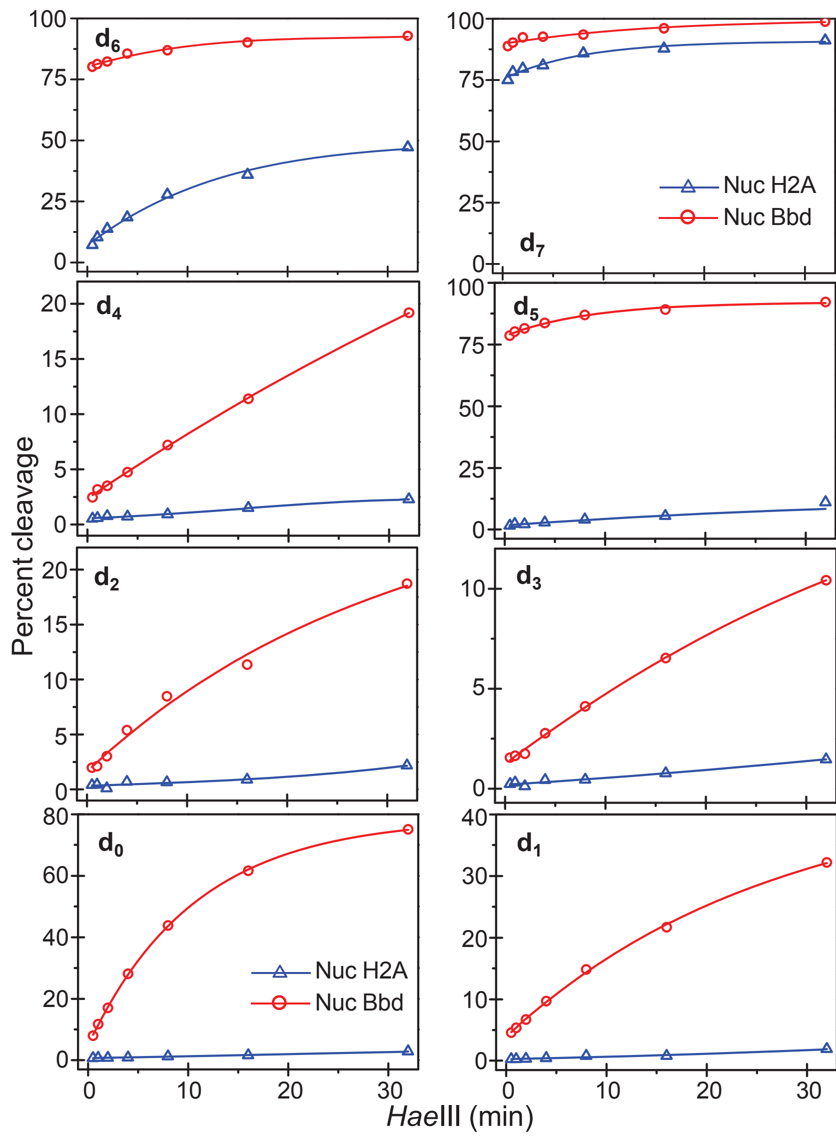


Figure S2

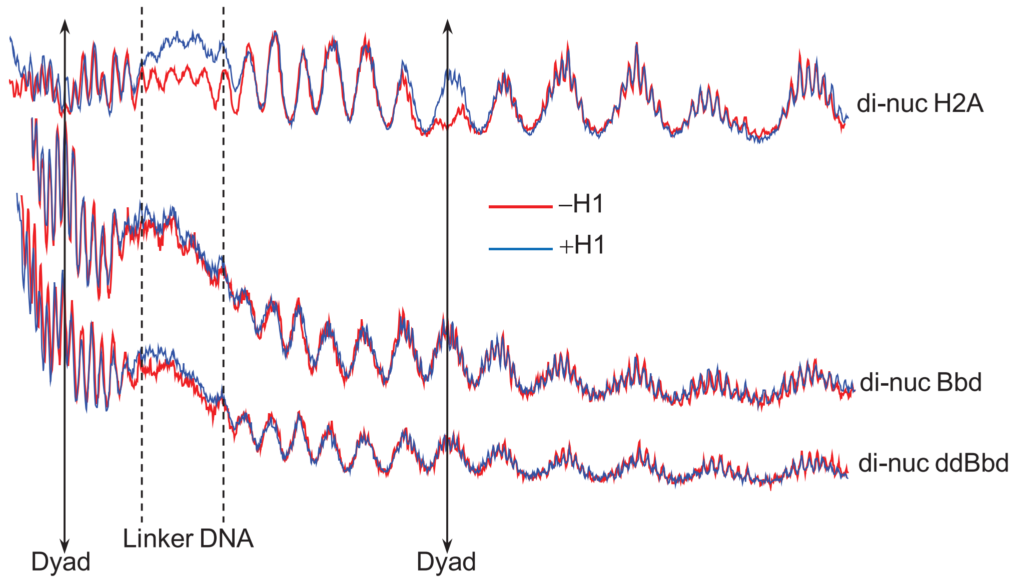


Figure S3