Role of ASF1 in Histone Deposition During Replication

By

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Abstract

Chromatin acts as a key element that controls access to the DNA. The assembly and desassembly of chromatin structure are of central importance for cellular functions. The main factors that determine chromatin assembly, reconfiguration and dynamics are histone chaperones and chromatin remodeling factors. Asf1 is a highly conserved histone chaperone that facilitates histone deposition and removal during nucleosome assembly and disassembly. Asf1 cooperates with the chromatin assembly factor 1 (CAF-1) to promote replication-dependent chromatin assembly and with HIRA to promote replication-independent chromatin assembly. Mammalian cells possess two isoforms of Asf1; Asf1a and Asf1b. However to date, whether they have individual functional roles has remained elusive. Here, we purified the epitope tagged Asf1a and Asf1b complexes from HeLa cells soluble nuclear extracts and analyzed their specific interacting partners. Mass spectrometry analysis followed by immunoblotting revealed that Asf1a associates exclusively with HP1⟨ and Asf1b with HP1®. The selectivity of this interaction can be recapitulated *in vitro* using highly purified recombinant proteins. Mutational analysis revealed that the two Asf1 isoforms discriminate between HP1⟨ and HP1® through their divergent C-terminal tail, which selectively recognizes an extended non-conserved Hinge domain encompassing the first 11 amino-acids of either HP1⟨ or HP1® chromoshadow domains. The interaction of Asf1 with the Hinge domain has important functional implications and might serve to counteract the binding of non-coding RNA to HP1. Our data elucidate for the first time the functional importance of Asf1 isoforms and reveal specific interactions with the different HP1 isoforms. Asf1a/b may associate with specific HP1 isoforms to promote nucleosome occupancy at defined chromatin loci.

Abbreviations

ASF1a: anti-silencing function 1a protein

Asf1b: anti-silencing function 1b protein

ATR: Ataxia telangiectasia and Rad3 related

bp : base pare

BrdU: Bromodeoxyuridine

CAF1: chromatin assembly factor 1

CENP-A: centromere protein A

CDK: Cyclin dependent kinase

DAXX: death-domain associated protein

DNA: deoxyribonucleic acid

dNTP: deoxyribonukleotide

DSB: double strand break

DTT: dithiothreitol

FACS: Fluorescence activated cell sorting

GST: Glutathione-S-transferase

HAT 1: histone acetyltransferase 1

HIRA: HIR histone cell cycle regulation defective homolog A

HJURP: holliday junction recognition protein

HP1: heterochromatin protein 1

HSP90: heat shock protein 90

HU: hydroxyurea

IPTG: Isopropyl-β-D-thiogalaktopyranosid

IP : Immunoprecipitation

MCM: minichromosome maintenance

NAP-1: nucleosome assembly protein-1

NCP: nucleosome core particle

Np : nucleoplasmin

PCNA: proliferating cell nuclear antigen

PCR: Polymerase chain reaction

PI3K: Phosphoinositide-3 kinase

Pol: Polymerase

Rad53: Radiation sensitive mutant 53

RC: replication-coupled

RI: replication- Independent

Rtt106: regulator of Ty1 transposition protein 106

sNASP: somatic nuclear autoantigenic sperm protein

S.cerevisiae: Saccharomyces cerevisiae

SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis

S.pombe: Schizosaccharomyces pombe

ssDNA: single-strand DNA

tNASP: testicular nuclear autoantigenic sperm protein

TLK1: Tousled-like kinase 1

TLK2: Tousled-like kinase 2

UBN1: Ubinuclein 1

WT : Wildtype

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INTRODUCTION

1.1   Chromatin

In eukaryotic cells, the genome is packed into chromatin, a hierarchically organized complex composed of DNA, histones and non-histone proteins. Chromatin can exist under different higher structures, in which DNA is more or less accessible to regulatory proteins. The first observations of eukaryotic interphase nuclei have shown the presence of two forms of chromatin; a condensed form named heterochromatin, and a more relaxed one named euchromatin (Lamond and Earnshaw, 1998). Heterochromatin is generally described as transcriptionally inactive, unlike euchromatin, which is associated with the active part of genome. However, all inactive genes and non-transcribed regions of the genome do not appear as heterochromatin. Furthermore, the genome-wide patterns of alternatively condensed chromatin persist through cell generation. Because of this property, chromatin is believed to be a principal carrier of the epigenetic information, which plays an important role in many physiological processes such as development. Aberrations in the maintenance and regulation of alternative chromatin states are being increasingly recognized as important factors in carcinogenesis and other pathologies.

1.1.1   Nucleosome

The fundamental building block of chromatin is the nucleosome, a histone octamer composed of two copies of each core histone protein, H2A, H2B, H3 and H4 with 147bp DNA wrapped in ~1.7 left handed super helical turns (Figure1.a) (Kornberg, 1974; Luger et al., 1997).

Each core histone displays a characteristic and highly conserved structure formed by three α-helixes linked by two short loops L1 and L2. This "histone fold" is used to form "handshake" arrangements between histones H2A and H2B, and histones H3 and H4 (Figure1.b).
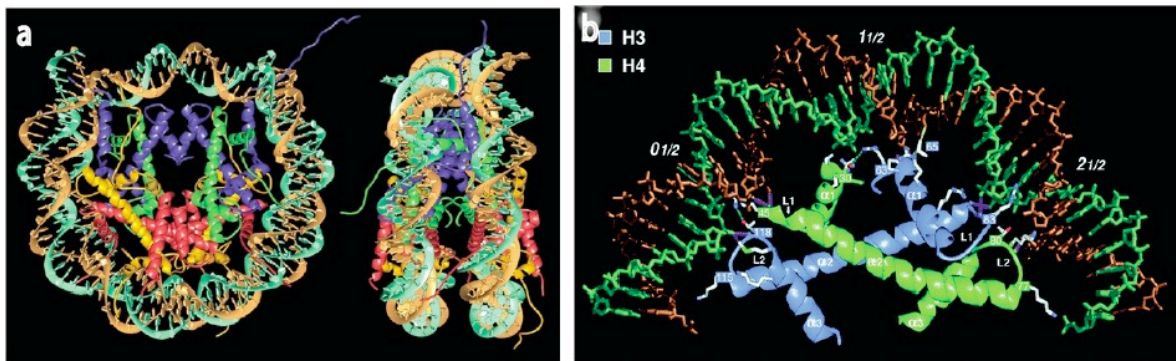
Figure 1:   a. Nucleosome core particle: ribbon traces for the eight core histones and 147bp DNA phophodiester backbones (blue: H3; green: H4; yellow: H2A; red: H2B; brown and turquoise: DNA

b. Ribbon traces of the H3-H4 histone-fold pair. The α1-L1-α2-L2-α3 structure is shown. (Adapted from Luger et al., 1997)

The protein octamer is divided into four dimers defined by H2A-H2B and H3-H4 pairs. The two H3-H4 dimers interact through a four α-helixes bundle formed by the two H3 histone folds to define the H3-H4 tetramer. Then, each H2A-H2B dimer interacts with the tetramer through a second four α-helixes bundle between H2B and H4 histone-folds (Arents et al., 1991).

All the core histones have N-terminal basically charged tails. These tails represent up to 20% of the mass of the core histones. They show no secondary structures and extend radially beyond the disc-shaped surface of the nucleosome, in position to associate with linker DNA or adjacent nucleosomes (Arents and

Moudrianakis, 1993; Luger et al., 1997). They also form ideal targets for covalent modifications. Two nucleosome core particles are separated by linker DNA varying in length from 10 to 80 bp. Histone H1 locks the linker DNA at the entry and exit points of the nucleosome (Maier et al., 2008; Ramakrishnan, 1997; Widom, 1998). Nucleosomes are connected with one another to form nucleosomal arrays which further fold into less understood higher order chromatin structures.

1.1.2   Chromatin higher order structures

Condensation has been shown to be an intrinsic property of the nucleosome arrays. The presence of linker histone H1 or its variant H5 helps compaction of the chromatin fiber and stabilizes various folding patterns (Carruthers et al., 1998). H1 winds the nucleosomes together into a chromatin fiber that is about 30 nanometers in diameter. These chromatin fibers are looped into a 300 nanometer coiled chromatin fiber, and these coiled chromatin fibers are
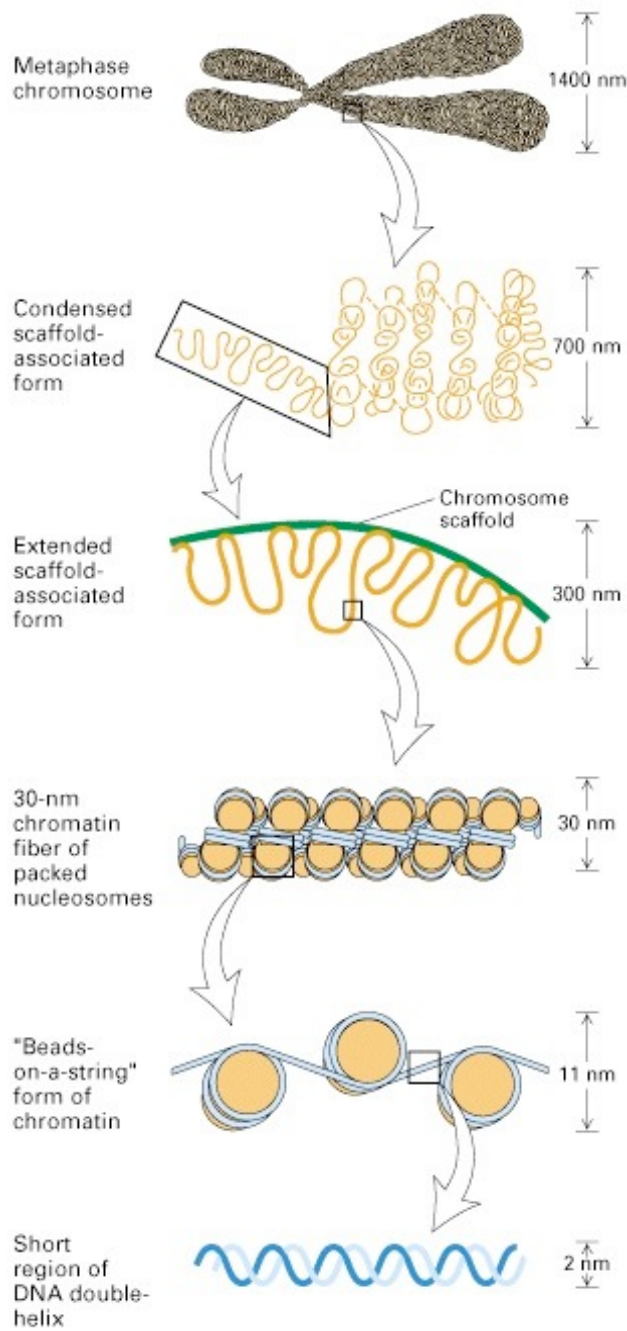
Figure 2: Hierarchical folding of chromatin (Lodish et.al., 2000)

then wound into 700 nanometer-condensed chromatin. A chromosome that has been observed during cell division has a diameter of 1400 nanometers (Figure 2) (Hamiche and Shuaib, 2012; Woodcock, 2006; Woodcock and Dimitrov, 2001). The chromatin fiber itself undergoes further levels of packaging resulting in compaction by a factor of

~1000 in interphase euchromatin and ~10,000 in heterochromatin (Khorasanizadeh, 2004).

1.1.3   Specialized chromatin structures

1.1.3.1 Euchromatin

On the basis of microscopic observations, chromatin is divided into two specialized structures, euchromatin and heterochromatin. Euchromatin replicates relatively early in the cell cycle is decondensed during interphase, and is transcriptionally more active than heterochromatin (Figure 3). In general, decondensed euchromatin is hyperacetylated. The acetylation of histone amino-terminal tails reduces the global charge of the tails, and allows them to interact with adjacent nucleosome, which destabilizes chromatin structure and finally facilitates the recruitment of transcription factors and co-factors (Annunziato et al., 1988; Nightingale et al., 1998). Studies have shown striking link between actively transcribed gene in euchromatin, like HSP70, and heterochromatin protein 1 (HP1) (Piacentini et al., 2003). In addition, three euchromatic genes Pros35 (encoding
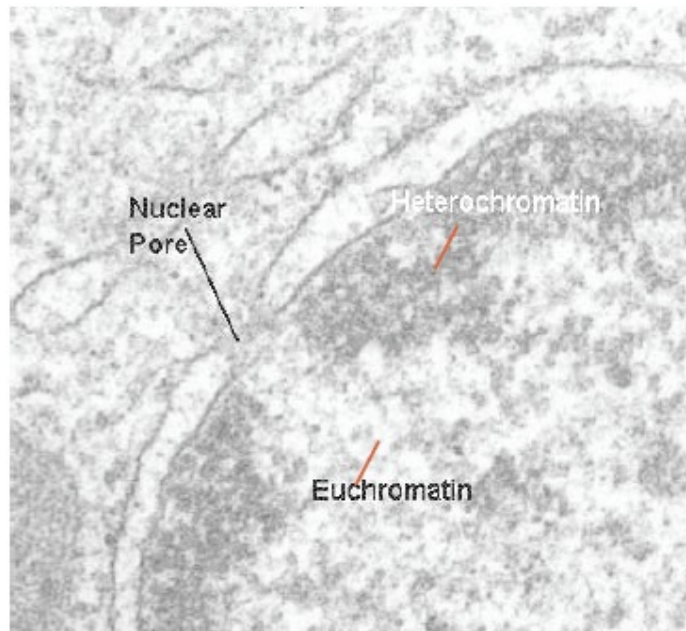
Figure 3. Electron Microscope (EM) of cell, indicating Euchromatin and Heterochromatin regions (Alberts et al., 1994).

the 35-kd subunit of the proteasome), cdc2 (encoding a cell cycle dependent protein kinase) and CG5676 (encoding a predicted protein of uncharacterized function), found to be associated with HP1 (Cryderman et al., 2005). Lately, around one hundred euchromatic genes are shown to be associated with HP1, leading towards a new functional link between euchromatin and heterochromatin (Piacentini and Pimpinelli, 2010).

1.1.3.2 Heterochromatin

Heterochromatin is the nuclear material distinguished by its dense pattern of staining throughout the cell cycle. Heterochromatin is not simply a DNA sequence, but rather a chromatin state. Heterochromatin is highly condensed, inaccessible and less transcribed (Figure 3) (Huisinga et al., 2006). Heterochromatin is the layer of different

kinds of information, including chromosomal location, nuclear localization and density of repetitive DNA elements (Birchler et al., 2000; Weiler and Wakimoto, 1995). The chromosomal regions that contain high density of repetitive DNA elements such as clusters of satellite sequences and transposable elements, which are found at centromeres, telomeres and "knobs" are the main targets of heterochromatin formation (Birchler et al., 2000).

The regions remaining condensed through the entire cell cycle are called constitutive heterochromatin. However, heterochromatin is also found at the developmentally regulated loci, where chromatin state can change in response to cellular signals and gene transcription. These regions are referred as facultative heterochromatin. Molecular characterization has led to the identification of several key constituents of heterochromatin. Extensive methylation of histone 3 at Lysine 9 is a characteristic of heterochromatin regions (Richards and Elgin, 2002). Other differences involve protein components of heterochromatin. Heterochromatin protein 1 (HP-1) is found abundantly in heterochromatin regions of fly and mammalian chromosomes (James and Elgin, 1986; Wreggett et al., 1994), and other proteins, such as SU(VAR)3-7 and SU(VAR)3-9 also contribute to the heterochromatic state (Aagaard et al., 1999; Reuter et al., 1990; Wu et al., 2005). However, it should be noted that some genes, termed heterochromatic genes, require the presence of HP1 for their expression (Lu et al., 2000), but the mechanism involved is not yet known.

1.1.3.3 Heterochromatin Protein (HP1⟨, HP1®, and HP1©)

Specific modifications of histones are essential epigenetic markers which are heritable changes in gene expression that do not affect the DNA sequence (Strahl and

Allis, 2000). Methylation of lysine 9 in histone H3 is recognized by HP1, which directs the binding of other proteins to control chromatin structure and gene expression (Lachner et al., 2001; Nakayama et al., 2001). Methylation at lysine 9 in histone by Suv39h1 methyltransferase strongly correlates to repressed chromatin state (Rea et al., 2000). HP1 was initially identified in *D. melanogaster* in a screen for nuclear protein that showed a predominant distribution to the chromocentre on polytene chromosomes (James and Elgin, 1986). This highly conserved protein has homologues in various organisms, ranging from *S. pombe* (Swi6) to mammals. In mammals three HP1 isoforms, HP1⟨, HP1® (also known as MOD1 or M31) and HP1© (also known as MOD2 or M32) have been identified (Singh et al., 1991). HP1 proteins are small (around 25 kDa) and contain a conserved N-terminal chromodomain (chromatin organization modifier), followed by variable hinge region and a conserved carboxy-terminal chromoshadow domain (Figure 4).

In general chromodomains are found in numerous proteins, many of them are known to function in chromatin organization and the regulation of gene expression such as polycomb proteins and Suv39h1. The three dimensional structure of both chromodomain (CD) and chromoshadow (CSD) domain of mouse HP1® shows that each domain forms a hydrophobic pocket and can function as protein-binding motif (Ball et al., 1997; Nielsen et al., 2002). HP1 serves as a bridging protein, connecting histones, through interactions with the CD, non-histone chromosomal proteins, through the interactions with the CSD. In mammals, the association of HP1, the retinoblastoma (Rb) protein and Suv39h1 with the cyclin E promoter correlates with gene silencing (Nielsen et al., 2001). Furthermore, HP1 has been implicated in gene repression mediated by Kruppel-associated box (KRAB) zinc finger proteins.
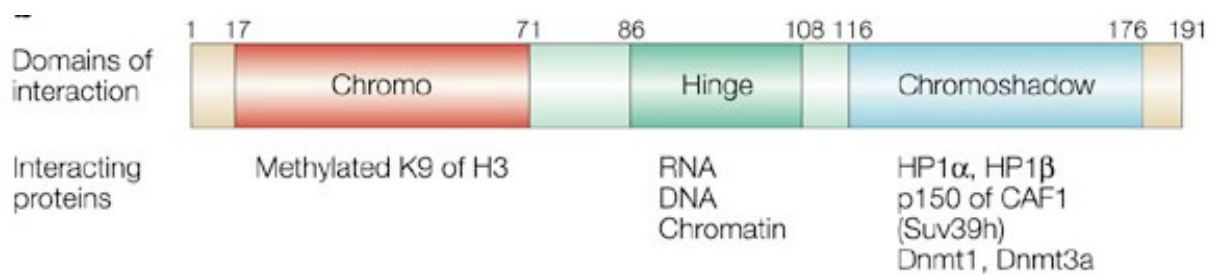
Figure 4. HP1⟨ domains of interaction with selected partners that are potentially important for the stability of heterochromatin domains (Maison et al., 2004).

Taken together these findings suggest that HP1 is recruited to specific gene by protein-protein interactions, resulting in gene silencing by an unknown mechanism. Regarding transcriptional regulators, HP1 interacts with proteins involved in DNA replication and repair. Chromatin assembly factor 1 (CAF1) that assembles histones H3 and H4 into newly replicated DNA in both euchromatic and heretochomatic regions of the genome. Although the significance of CAF1-HP1 interaction is not clear, the data suggest that CAF1 might stabilize heterochromatin structure during chromosomes decondensation and transcription. In mammals, HP1 phosphorylation status changes through the cell cycle. HP1⟨ and HP1© exhibit increased level of phosphorylation during mitosis (Minc et al., 1999). Clearly, the role of phosphorylation needs further investigation to understand the biological significance of this dynamic process. Recently a detailed study showed that HP1 has preferred binding affinity for trimethylated K9 of histone H3 (H3K9me3) (Fischle et al., 2003b). The histone methyltransferase, Suv39h1 is responsible for (H3K9me3) modification and is able to bind to HP1 (Aagaard et al., 1999). HP1 proteins can bind to RNA *in vitro*, but this binding does not involve the HP1 chromodomain (Muchardt et al., 2002). In addition, the hinge region of HP1 can

bind DNA without obvious sequence specificity (Meehan et al., 2003; Sugimoto et al., 1996). The chromoshadow domain of HP1⟨ functions as a dimerization module and mediates the interaction with either HP1® or HP1⟨ itself (Le Douarin et al., 1996). HP1 interacting partners include the DNA methyltranferases Dnmt1 and Dnmt3a, which are involved in CpG methylation (Fuks et al., 2003). HP1 chromoshadow domain binds the large subunit p150 of chromatin assembly factor-1 (CAF-1) a factor that is implicated in histone deposition during both DNA replication and repair (Mello and Almouzni, 2001; Murzina et al., 1999). Therefore, the role of HP1 is proposed to be dependent on the presence of various binding partners, and it could possibly exchange the partners and contribute to a structure, which can be highly dynamic.

1.2   Chromatin dynamics

The DNA wrapped in nucleosomes is inaccessible to the proteins that bind to it and regulate transcription, replication, recombination, and repair. Therefore remodelers are needed to provide regulated DNA accessibly in packaged regions. There are chromatin-remodeling ATPase, histone modifying enzymes, linker histones, transcription factors and the nature of the DNA sequence are responsible for making the chromatin more or less accessible. Transcription factors, linker histones, and enzymes that act on chromatin may themselves show dynamic interactions with the chromatin template, thus allowing rapid changes of chromatin states (Karpova et al., 2004; Kassabov et al., 2003).

1.2.1 Histone post-translational modifications

One major challenge in chromatin biology is connecting particular histone post-translational modifications (HPTMs) with different biological functions and vice versa. Histone post-translational modifications (HPTMs) play a crucial role in changing the chromatin structure by both directly or indirectly activating chromatin remodeling complexes. HPTMs have been shown to control several developmental processes, and are deregulated in many human diseases, including cancer (Mathews et al., 2009). Histones are subjected to a variety of post-translational modifications, including acetylation (Grunstein, 1997; Sterner and Berger, 2000), methylation (Zhang and Reinberg, 2001), ubiquitination (Davie and Murphy, 1990), phosphorylation (Nowak and Corces, 2004), sumoylation (Nathan et al., 2003), ADP ribosylation (Adamietz and Rudolph, 1984), glycosylation (Liebich et al., 1993), biotinylation (Hymes et al., 1995), and carbonylation (Wondrak et al., 2000).

Figure 5: Post-translational modifications of core histones (Bhaumik et al., 2007). (ph-Phosphorylation, me-methylation, ac-acetylation, ub- ubiquitination).

One of the best understood histone modifications, in that aspect, is histone acetylation (Grunstein, 1997; Turner, 2000). Most of the post-translational modifications take place within the tail extensions that protrude from the nucleosome. HPTMs might affect electrostatic interactions between the histone tails and DNA to "loosen" chromatin structure. Later, it was proposed that combinations of these modifications might create binding epitopes to recruit other proteins. Recently, more modifications have been found in the structured regions of histones, and these modifications may affect histone-DNA and histone-histone interactions within the nucleosome core (Hansen et al., 1998; Wolffe and Hayes, 1999). Phosphorylation, particularly histones H1 and H3 has long been implicated in chromosome condensation during mitosis (Bradbury, 1992; Koshland and Strunnikov, 1996).

1.2.1.1 Acetylation

Histone acetylation and deacetylation of the lysine residues present in the histone tails has long been linked to transcriptional activity (Allfrey et al., 1964). Indeed, histone acetylation is strongly associated with active transcription and histone acetylation sites are required for gene activity (Shahbazian and Grunstein, 2007). There are twelve known modification sites two on histone H2A (K5, K9), two on histone H2B (K12, K15), four on histone H3 (K9, K14, K18, K56) and four on histone H4 (K5, K8, K12, K16) (Leszinski et al., 2012; Ohzeki et al., 2012; Roth et al., 2001). It is believed that acetylation may affect chromatin structure as it neutralizes the basic charge of lysine, which may affect the interaction of DNA with histones and nucleosome-nucleosome interactions (Tse et al., 1998). It has been shown that acetylation of H4K16

has a negative effect on the formation of the 30 nm chromatin fiber (Shogren-Knaak et al., 2006). Thus, histone acetylation results in a 'loosening' of chromatin structure to allow greater access to transcription factors. Furthermore, histone H3 K9 acetylation in promoter regions is associated with low nucleosome density in the vicinity of transcription start sites (Nishida et al., 2006). Histone acetylation can also function as recognition sites for factors that promote transcription. For example, the bromodomain of BRG1, which is the catalytic subunit of the SWI/SNF chromatin remodeling complex, binds acetylated H4K8, while acetylation of H3K9 and H3K14 is critical for the recruitment of TFIID (Agalioti et al., 2002). Recent studies, which mapped acetylated histones on a genome-wide level, found that acetylation of most lysines in the histone H3 and H4 tails was observed in the 5' end of coding regions and correlated with active transcription (Kurdistani et al., 2004; Pokholok et al., 2005; Roh et al., 2004; Roh et al., 2007). Furthermore, many inducible genes are marked by histone acetylation even in the inactive state suggesting that the presence of histone acetylation serves to prime these genes for activation at a later stage (Roh et al., 2004).

1.2.1.2   Methylation

Histones can be methylated either on lysine (K) or arginine (R) residues (Murray, 1964; Patterson and Davies, 1969). Lysine residues can be mono-, di-, or tri-methylated (Lachner et al., 2003), whereas arginine residues can only be mono- or di-methylated but di-methylation can occur in a symmetrical or asymmetric configuration (Yuan et al., 2011; Zhang, 2004). Histone H3 can methylated on a number of lysine sites, which include K4, K9, K27, K36 and K79. Histone H3 can also be methylated on a number of arginine sites: R2, R8, R17 and R26. On histone H4, the main sites of

methylation are K20 and R3. Methylation at H3K4 was first observed in the trout testes (Honda et al., 1975), and several studies have linked it to active gene expression in numerous eukaryotes (Leszinski et al., 2012; Ng et al., 2003; Santos-Rosa et al., 2002; Strahl et al., 1999; von Holt et al., 1989). Heterochromatin in higher eukaryotes is characterized by histone hypoacetylation and H3K9 methylation (Richards and Elgin, 2002). Heterochromatin protein 1 (HP1) was shown to specifically recognize methylated H3K9 via its chromodomain (Lachner et al., 2001; Nakayama et al., 2001). This recognition of H3K9 by HP1 is required for the formation of heterochromatin. However, H3K9 methylation and HP1 binding were recently detected on active genes (Vakoc et al., 2005; Vakoc et al., 2006), suggesting that H3K9 methylation is not limited to inactive regions of chromatin. Methylation of histone H3K27 exhibits some similarities to K9 methylation. Both lysines are found within an ARKS sequence on histone H3 and K27 methylation and are associated with transcriptional silencing (Ringrose et al., 2004). In particular, methylation of H3K27 is characteristic of the inactive X chromosome in female cells (Mak et al., 2002; Wang et al., 2001). Methylation of H3K79 is unusual because this modification lies at the core of the nucleosome rather than on the tail. Global analysis of H3K79 methylation has shown that this modification primarily associates with the coding region of actively transcribed genes (Miao and Natarajan, 2005). H3K79 methylation has also been implicated in DNA repair considering that the checkpoint protein p53BP1 has been shown to bind methylated H3K79 (Martin and Zhang, 2005). H4K20 methylation is connected to transcriptional repression and DNA repair, although very little is known about how it functions in these processes.

1.2.1.3 Phosphorylation

Histone phosphorylation is also linked to the regulation of chromatin structure. The role of histone phosphorylation had been implicated in different processes such as transcription, DNA repair, apoptosis and chromosome condensation (Cheung et al., 2000). Most studies have focused on the role of H3S10 phosphorylation (Johansen and Johansen, 2006). Phosphorylation of this residue was found to occur in tandem with the activation of immediate early genes such as *c-jun* and *c-fos* (Mahadevan et al., 1991), and at activated heat shock genes. Phosphorylation of H3S10 was also observed during chromosome condensation. H3S10 seems to function by regulating a methylation/phosphorylation switch that inhibits HP1 binding to H3K9me3 (Fischle et al., 2003a), and indeed this was shown to be true as phosphorylation of H3S10 is responsible for HP1 dissociation during mitosis (Fischle et al., 2005). These observations suggest a model for how H3S10 phosphorylation functions in the two opposing processes of gene activation and chromosome condensation. During interphase this modification promotes removal of HP1 from specific regions, allowing gene expression. Removal of the phosphorylation mark would therefore promote heterochromatin formation and promote chromatin condensation.

1.2.1.4 Ubiquitination

In human, histones H2A and H2B have been reported to be ubiquitinated, at K119 in H2A and K120 in H2B. Histone H2A was the first ubiquitinated histone to be identified (Goldknopf et al., 1975), and the majority of this modification is the monoubiquitinated form. H2A ubiquitination has been linked to polycomb silencing and

X-chromosome inactivation (Wang et al., 2004). Ubiquitinated H2A at K119 was found on the inactive X-chromosome in females and is correlated with the recruitment of PcG proteins PRC1-like (PRC1-L). The ubiquitin moiety is approximately half the size of a core histone, so it has been suggested that ubiquitination of a nucleosome would impact chromatin folding, thus affecting transcription (Shilatifard, 2006).

1.2.1.5   Sumoylation

SUMO is a small ubiquitin-related protein of ~100 amino acids, which is capable of being ligated to its target protein. Protein sumoylation is involved in the regulation of transcription factors and components of the transcriptional and often results in transcriptional repression (Hannich et al., 2005; Manza et al., 2004). Histone H4 sumoylation has been reported in mammalian cells and correlates with transcriptional repressive events such as histone deacetylation and HP1 recruitment (Shiio and Eisenman, 2003).

1.2.1.6   ADP-ribosylation

Mono-ADP ribosylation of histones is linked to DNA repair and cell proliferation (Hassa et al., 2006). Histones are mono-ADP-ribosylated when exposed to DNA damaging agents. This modification has the potential for 'cross-talking' with other modifications such as mono-ADP-ribosylation on H4 occurs preferentially when H4 is acetylated (Golderer and Grobner, 1991). Poly-ADP-ribosylation has not been confirmed on histones but it may play a role in local chromatin compaction.

1.2.2 Histone variants

Additional complexity has recently been added to chromatin dynamics with the renewed interest in alternative versions of the canonical core histones. These paralogues, now known as histone variants, share the same overall structure with the canonical histones, displaying for instance the histone fold, but differ in terms of primary sequence from their canonical relatives. Variant histones have been found in all eukaryotes and seem to play particular roles in the specific regions of the genome. Furthermore, at least some of the variant histones appear to be incorporated at specific sites, replacing canonical histones. The substitution of a canonical histone by a variant will then create a new nucleoprotein complex, with an alternate structure and stability. Some histone variants are interesting candidate for an epigenetic marking of the genome.

1.2.2.1   Histone H1 variants

Histone H1 has a role in stabilizing both the nucleosome and chromatin higher-order structure. H1 in humans is a family of closely related, single gene encoded proteins, including seven somatic subtypes (H1.1, H1.2, H1.3, H1.4, H1.5, H1.0, and H1X), three testis-specific variants (H1t, H1T2m and HILS1), and one restricted to oocytes (H1oo) (Henikoff and Ahmad, 2005; Szenker et al., 2011; Yun et al., 2011). Histone H1 is a group of one of the most well studied histone variants till date. They are about 21 to 29 kDa proteins consisting of 194-225 amino acid residues depending on the variants. Among the somatic histone H1 variants, H1.1 to H1.5 are expressed in a

replication-dependent manner, whereas H1.0 and H1X are replication- independent. The H1.1 to H1.5 encoding genes are clustered in a region of chromosome 6 together with the core histone genes, whereas H1X and H1.0 genes are on chromosome 3 and 22 respectively. Change in the relative amounts of the somatic H1 variants and their potential roles during differentiation have been little investigated. The histone H1 consists of a well-conserved hydrophobic GH1 domain but less conserved N- and C-terminal parts. H1 variants are highly similar between human and mouse with at least 90% sequence identity with in the GH1 domain (Globular domain of histone H1). GH1 consists of three helices and is primarily involved in DNA binding (Clark et al., 1993; Ramakrishnan et al., 1993; Varga-Weisz et al., 1994). The terminal parts are thought to be involved in binding of non-histone chromatin proteins regulating transcription (Hansen et al., 2006; Zlatanova et al., 2000). The C-terminal tail of the linker histone is involved in chromatin condensation and H1-dependent activation of the apoptotic nucleases (Widlak et al., 2005). The mode of binding of histone H1 to the linker DNA of nucleosome fiber is unclear. Similarly to the core histones, the linker histones are the components of both condensed and transcriptionally active chromatin. Diverse posttranslational modifications such as acetylation, methylation, and phosphorylation are considered to be regulators of histone H1 function in chromatin condensation and chromatin specific proteins.

1.2.2.2   Histone H2A variants

H2A variants are particularly divergent in regions that are involved in histone-histone contacts or are located as surface residues. Four histone H2A variants have been reported in mammals (H2A.Z, H2A.X, marcoH2A, and H2A.Bbd). H2A.Z is highly

conserved throughout evolution and it has a single evolutionary origin and remains distinct from all other H2A variants (Malik and Henikoff, 2003). H2A.Z has a distinct function from all other H2A variants. H2A.Z differs from canonical H2A and other H2A variants mainly in its "docking" domain in the C-terminus and in the L1 loop where two H2A molecules interact (Arents et al., 1991; Luger et al., 1997; Suto et al., 2000). The SWR1 complex, which is an ATP- dependent chromatin-remodeling complex, was the first identified H2A.Z chaperone that mediates H2A.Z deposition. Nap1 mediates the nuclear import of the cytosolic H2A.Z-H2B, whereas Chz1 presents the H2A.Z-H2B dimers to the SWR1 complex for the final deposition. SWR1 replaces H2A-H2B with H2A.Z-H2B in a stepwise manner, one copy of the H2A.Z-H2B dimer at a time, which leads to the formation of intermediate hybrid nucleosomes that contain both H2A and H2A.Z (Luk et al., 2010; Straube et al., 2010). The role of H2A.Z in heterochromatin formation and maintenance is first enriched at pericentromeric heterochromatin and subsequently enriched at other chromatin regions. H2A.Z directly interacts with the pericentromeric heterochromatin protein INCENP *in vivo* (Rangasamy et al., 2003). H2A.Z depletion caused genomic instability and disruption of HP1⟨ localization at the pericentromeric regions, which suggests that HP1⟨ functions and pericentromeric heterochromatin identity are regulated by H2A.Z during the early embryonic development in mice (Rangasamy et al., 2004).

H2A.X variant is present nearly in all eukaryotes and this histone variant has a histone fold domain that is similar to the canonical histone H2A. H2A.X has a unique C-terminal motif termed SQ (E/D)√ (where √ represents a hydrophobic residue) (Talbert and Henikoff, 2010). This motif can become rapidly phosphorylated by the phosphoinositide 3-kinase-like kinases, ataxia telangiectasia mutated (ATM), ataxia telangiectasia and RAD3-related (ATR) and DNA-dependent protein kinase (DNA-PK).

This phosphorylated form is known as ©H2A.X. Phosphorylation of ©H2A.X occurs in response to double-strand breaks (DSBs), at which ©H2A.X helps to recruit DNA repair proteins and histone modifying enzymes (van Attikum and Gasser, 2009). H2A.X has also been implicated to play a role in meiosis, growth, tumor suppression, and immune receptor rearrangements (Fernandez-Capetillo et al., 2004; van der Heijden et al., 2007).

MacroH2A is vertebrate specific H2A variant that contains two distinct domains. N-terminal region of macroH2A is similar to canonical H2A and C-terminal region contains large (200 residue) called "the macro domain", which does not have sequence similarity with any other histones (Malik and Henikoff, 2003; Pehrson and Fried, 1992). In mammals, macro H2A is enriched on the inactive X chromosomes in females and thought to be an epigenetic marker of X-inactivation (Costanzi and Pehrson, 1998; Mermoud et al., 1999; Rasmussen et al., 2000). Several studies have suggested potential mechanisms where macroH2A represses gene expression. It has been shown that the C-terminal macro domain of macroH2A interferes with the binding of transcription factors, and the N-terminal domain is involved in chromatin remodeling via SWI/SNF (Angelov et al., 2003).

H2A.Bbd is the most recently discovered H2A variant, which has a truncated C-terminal docking domain. It was named H2A.Bbd because of its unique genomic distribution, where it is deficient in inactive X chromosomes (bar body deficient). The H2A.Bbd nucleosome binds only 116 base pairs of DNA and is less stable than the canonical nucleosome (Bao et al., 2004; Chadwick and Willard, 2001; Gautier et al., 2004). H2A.Bbd lacks a small acidic region on the nucleosome surface that is involved in transcriptional repression and also lacks K119, which is often ubiquitinated on canonical H2A at transcriptionally inactive regions. The unique subnuclear localization and key features of H2A.Bbd suggest that it is involved in gene activation.

1.2.2.3   Histone H3 variants

To date, eight different histone H3 variants have been found in mammals: H3.1, H3.2, H3t, H3.3, H3.5, H3.X, H3.Y, and CENP-A. H3.3, CENP-A, H3.X and H3.Y are somatic histone variants, while H3t and H3.5 are testis specific variants (Hamiche and Shuaib, 2012; Postberg et al., 2010; Wiedemann et al., 2010). On the basis of their incorporation into chromatin they can be categorized into two categories: (i) canonical, replication dependent H3 histones (H3.1 and H3.2) and (ii) replication independent histone H3 variants (H3t, H3.3, CENP-A, H3.X, H3.Y and H3.5) (Hamiche and Shuaib, 2012). The CENP-A histone H3-like variants are centromere specific histones found in all eukaryotes from budding yeast to human. Genetic studies revealed that mammalian CENP-A and its counterparts in other eukaryotes (generally referred to as CenH3) are absolutely required for assembly of the kinetochore to which the spindle microtubules attach at mitosis and meiosis (Blower and Karpen, 2001; Buchwitz et al., 1999; Howman et al., 2000). Centromere is the chromosomal region that directs kinetochore assembly during mitosis in order to facilitate the faithful segregation of sister chromatids. In most eukaryotes, centromeres exist as a single locus on each chromosome, and a chromosome lacking a centromere will fail to segregate properly. Segregation errors lead to aneuploidy, which in turn causes cellular stress and greater genomic instability. The evolutionary conserved mark of centromere is the presence of unique nucleosome in which canonical histone H3 is replaced by CENP-A (Cse4 in budding yeast, Cnp1 in fission yeast, and CID/CenH3 in fruit flies). The essential constitutive centromere components include CENP-A (Howman et al., 2000), CENP-C (or Mif2 in budding yeast) (Fukagawa et al., 1999; Meluh and Koshland, 1995), CENP-

I (or Mis6 in fission yeast) (Goshima et al., 1999; Nishihashi et al., 2002), CENP-H (Fukagawa et al., 2001) and Mis12, which are required for the recruitment of many constitutive centromeric components. Furthermore, heterogeneity of the N-terminal domains in the CENP-A family could be the result of the differences in the chromosome segregation mechanisms between yeast and human.

1.2.2.4   H2B variants

Unlike the H3 and H2A histones, which have several variants with different functions, no ubiquitously expressed H4 and H2B variants have been reported. Nevertheless, a few tissue-specific H2B isoforms have been reported, including sperm specific H2B variant (spH2B), testis specific H2B variants, TH2B and H2BFWT (Gineitis et al., 2000).

1.2.3   ATP-dependent chromatin remodeling

ATP-dependent chromatin remodeling enzymes use energy from ATP hydrolysis to remodel nucleosomes (Narlikar et al., 2002). Remodeling enzymes disrupt histone-DNA interactions and they provide nucleosome "sliding", allowing transcription factors to access the DNA (Becker, 2002). They can also reposition the DNA so that it is accessible on the surface of the histone octamer (Aoyagi et al., 2002). In addition to these activities, remodeling complexes can transfer a histone octamer from one DNA template to another and can cause changes in super-helicity by twisting the DNA, which disrupts histone-DNA interactions (Gavin et al., 2001; Whitehouse et al., 1999). There are four classes of ATP-dependent remodelers, that can be

distinguished by their unique flanking domains: the SWI/SNF (Switch/Sucrose non-fermentable), the CHD (chromodomain and helicase-like domain), the ISWI (imitation SWI), and the INO80 families. All remodeler catalytic subunits share conserved ATPase domain.

1.2.3.1   SWI/SNF family remodelers

The SWI/SNF family remodelers were initially purified from *S. cerevisiae* and are composed of 8-14 subunits. Most eukaryotes utilize two related SWI/SNF family remodelers, built around two catalytic subunits. The catalytic ATPase includes an HSA (helicase-SANT), a post-HSA, and a C-terminal bromodomain (Mohrmann and Verrijzer, 2005). Other conserved subunits bear addition conderved domains: for example hBAF155/170 (SANT, SWIRM), and hBAF60 (SwiB), and human poly bromo (multiple bromodomains). This family has many activities, and it slides and ejects nucleosomes at many loci for different physiological processes but lacks role in chromatin assembly.

1.2.3.2   ISWI family remodelers

The ISWI family remodelers contain 2 to 4 subunits. dNURF, dCHRAC, and dACF complexes were initially purified from *D. melanogaster* and later hWICH or hNoRC.(Corona and Tamkun, 2004). Most eukaryotes built multiple ISWI family complexes using one or two different catalytic subunits, with specialized proteins. A set of domains bind to the C-terminus of ISWI family ATPase: aSANT domain (ySWI3, yADA2, hNCoR, hTFIIIB) adjacent to a SLIDE domain (SANT-like ISWI), which

together form a nucleosome recognition module that binds to an unmodified histone tail and DNA (Boyer et al., 2004). These proteins contain many domains including DNA-binding histone fold motifs (hCHRAC 15-17), plant homeodomain (PHD), bromodomains (hBPTF and hACF1) and additional DNA-binding motifs HMGI (Y) for dNURF301.

1.2.3.3 CHD family remodelers

The CHD family remodelers combine 1 to 10 subunits and were first purified from *Xenopus laevis*. Characteristic features include two tandemly arranged chromodomains on the N-terminus of the catalytic subunits. The catalytic subunit is monomeric in lower eukaryotes but can be in large complexes in vertebrates (Marfella and Imbalzano, 2007). Proteins often bear DNA-binding domains and PHD, BRK, CR1-3, and SANT domains. Some CHD remodelers slide or eject nucleosomes to promote transcription.

1.2.3.4 INO80 family remodelers

The INO80 (inositol requiring 80) family remodelers contain more than 10 subunits, include the SWR1-related complexes, and were initially purified from *S. cerevisiae* (Bao and Shen, 2007). Higher orthologs include hINO80, hSRCAP (SNF2-related CREB-activator protein), and p400, which also contain HAT activity. Both yINO80 and ySWRI complexes also contain actin and Arp4. INO80 has diverse functions, including promoting transcriptional activation and DNA repair. Although highly related to INO80, SWRI is unique in its ability to restructure the nucleosome by

removing canonical H2A-H2B dimers and replacing them with H2A.Z-H2B dimers, thereby inserting the histone H2A variant H2A.Z.

1.2.4 Histone chaperones

Three decades have passed since the discovery of nucleosomes in 1974 and the first isolation of histone chaperone in 1978. Nucleosomes can be reconstituted from DNA and histones by salt gradient dialysis (Axel et al., 1974; Kornberg and Thomas, 1974; Oudet et al., 1975). However the mixtures of histones and DNA are aggregated and precipitated at physiological ionic strength. Laskey et al constructed a cell-free nucleosome assembly system at physiological ionic strength with *Xenopus egg* extracts and used this system to isolate nucleoplasmin as a nucleosome assembly factor that does not require low-molecular-weight cofactors, such as ATP (Laskey et al., 1978). They termed this factor as "histone chaperone" because it prevents nonspecific interactions between DNA and histones and promotes specific interactions that lead to nucleosome assembly (Laskey and Earnshaw, 1980; Laskey et al., 1978). Later work showed that the slow addition of histone made it possible to assemble nucleosomes without the histone chaperone at near physiological ionic strength. Under these conditions, nucleosome assembly could be stimulated by the addition of other chromatin components, like linker histone H1 and non-histone protein HMG (Bonne-Andrea et al., 1984; Drew, 1993), acidic polymers (Stein et al., 1979), and RNA (Nelson et al., 1981).

These studies resulted in the isolation of other histone chaperones and demonstrated that there are various types of histones chaperones (Table 1) (Loyola and Almouzni, 2004). Biochemical studies have revealed that histone chaperones interact not only with histones but also with numerous other chromatin factors.
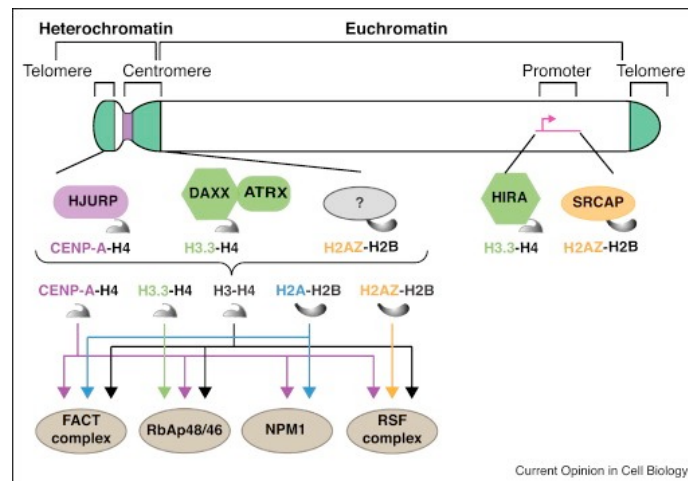
Figure 6: Distribution of histone chaperones at mammalian centromeres (Boyarchuk et al., 2001).

Histone chaperones include Nucleosome Assembly Protein 1 (NAP1) (Ishimi et al., 1984; Ito et al., 1996), Chromatin Assembly Factor 1 (CAF1), N1/N2 (Bonner, 1975; Kleinschmidt et al., 1986), Nucleoplasmin (Arnan et al., 2003; Laskey et al., 1978; Prado et al., 2004), HIRA (Ray-Gallet et al., 2002), DAXX (Drané et al., 2010) and ASF1 (Tyler et al., 1999; Tyler et al., 2001). Histone chaperones are localized at different sites of the genome (Figure 6). Interactions between histone chaperones and chromatin factors are important for the biochemical and biological functions of histone

Classification of chaperones		Histone chaperones in species of function identification		Histone selectivity	Conservation	Main functions
Class I: single chaperone	H3-H4	Asf1 (D.m.)[126]		Both H3.1-H4 & H3.3-H4	S.c., Asf1; S.p., Cia1 A.t., Sga1 & Sga2; D.m., Asf1; X.l., Asf1; M.m., Asf1a & Asf1b; H.s., Asf1a & Asf1b	Histone donor for CAF-1 and HIRA
		Fkbp39p (S.p.)[127]		H3-H4	S.c., Fpr; S.p., Fkbp39p	rDNA silencing
		HIRA (X.l.)[11]		H3.3-H4	S.c., HIR1/HIR2; S.p., Hip1, Sim2 A.t., HIRA; D.m., HIRA; X.l., HIRA; M.m., HIRA; H.s., HIRA	Deposition factor independent of DNA synthesis
		N1/N2 (X.l.)[128]		H3-H4[b]	X.l., N1/N2; M.m., tNasp & sNasp; H.s., tNasp & sNasp	H3-H4 storage in X. laevis oocytes
		Spt6 (S.c.)[129]		H3-H4	S.c., Spt6; S.p., Spt6 D.m., Spt6; X.l., Spt6; M.m., Spt6; H.s., Spt6	Transcription initiation & elongation
		Rtt106 (S.c.)[130]		H3-H4	S.c., Rtt106; S.p., SPAC6G9.03c	Heterochromatic silencing
	H2A-H2B	Nucleoplasmin (X.l.)[131]/ Nucleophosmin (NPM1) (H.s.)[132]		H2A-H2B	D.m., Nip; X.l., Nucleoplasmin; M.m., NMP1, NMP2 & NMP3; H.s., NMP1; NPM2 & NPM3	Storage in X. laevis oocytes, cytosolic-nuclear transport, replication, transcription
		Chz1 (S.c.)[16]		H2AZ-H2B	S.c., Chz1; H.s., HIRIP3?	Chz1: H2AZ incorporation by SWR1
		Nap1 (X.l.)[131] Nap1-related proteins: Nap1L2 (M.m.)[133], SET/TAF1b (H.s.)[134], CINAP (H.s., M.m.)[135] and Vps75 (S.c.)[136]		H2A-H2B[b]	S.c., Nap1; S.p., Nap1 & Nap1.2 A.t., NRP1 & NRP2; D.m., Nap1 X.l., Nap1; M.m., Nap1; H.s., Nap1	Cytosolic-nuclear transport Transcription Replication
		Nucleolin (H.s.)[25]		H2A-H2B[c]	S.c., NSR1 A.t., Nucleoli; X.l., Nucleolin M.m., Nucleolin; H.s., Nucleolin	Transcription elongation; assist chromatin remodelling
Class II: multi-chaperone complex	H3-H4	CAF-1 complex (H.s.)[8]	p150	H3.1-H4	S.c., Rlf2/Cac1; S.p., SPBC29A10.03C A.t., Fas1 D.m., p180; X.l., p150; M.m., p150; H.s., p150	Deposition factor coupled to DNA synthesis: -Replication -Repair
			p60	H3.1-H4	S.c., Cac2; S.p., SPAC26H5.03 A.t., Fas2; D.m., p150; X.l., p60; M.m., p60; H.s., p60	
			RbAp48[a]	H3-H4	S.c., Msi1/Cac3; S.p., Msi16 A.t., Msi1; D.m., p55; X.l., p48; M.m., RbAp48; H.s., RbAp48	
	H2A-H2B	FACT complex (H.s.)[24]	Spt16	H2A-H2B	S.c., Spt16; S.p., Spt16 A.t., Spt16; D.m., Spt16; X.l., Spt16; M.m., Spt16; H.s., Spt16	Transcription elongation
			SSRP1	H3-H4[d]	S.c., Pob3; S.p., Pob3 A.t., SSRP; D.m., SSRP1; X.l., SSRP1; M.m., SSRP1; H.s., SSRP1	
Class III: within enzymatic complexes	H3-H4	Chaperone	Enzymatic complex			
		Hif1 (S.c.)[137]	Hat1p/Hat2 (S.c.)	H3-H4	S.c., Hif1	Assist HAT
		Rsf-1 (H.s.)[138]	RSF (H.s.)	H3-H4	H.s., Rsf-1; M.m., Rsf-1	Assist RC
	ND/ multiple	Arp4 (S.c.)[15]	RC INO80 (S.c., D.m., M.m., H.s.) RC SWR1 (S.c.) HAT NuA4 (S.c.) RC+HAT Tip60 (D.m., M.m., H.s.)	ND	S.c., Arp4; S.p., Alp5 A.t., Arp4; D.m., BAP55; M.m., BAF53; H.s., BAF53	Assist RC
		Arp7, Arp9 (S.c.)[139]	RC SWI/SNF & RSC (S.c.): BAF/PBAF & BAP (D.m., M.m., H.s.)	ND	S.c., Arp7,9 A.t., Arp7,9; D.m., BAP55; M.m., BAF53; H.s., BAF53	Assist RC
		Arp8 (S.c.)[113]	RC INO80 (S.c., D.m., M.m., H.s.)	H3-H4	S.c., Arp8 D.m., BAP55; M.m., BAF53; H.s., BAF53	Assist RC
		Acf1 (D.m.)[140]	RC ACF/CHRAC (S.c., D.m., M.m.)	Both H2A-H2B & H3-H4	S.c., Itc1 D.m., Acf1; M.m., Acf1; H.s., Acf1	Assist RC
Multiclass chaperone	H3-H4	RbAp48[a] (H.s.)[8]	1. Single chaperone 2. CAF-1 complex 3. RC, HAT, HDAC, PC	Both H3-H4 & CENPA-H4	S.c., Msi1/Cac3; S.p., Mis16 A.t., Msi1; D.m., p55; X.l., p48; M.m., RbAp48; H.s., RbAp48	1. Centromeric chromatin maintenance 2. Chromatin assembly coupled to DNA synthesis 3. Assist multiple enzymatic activities

Table1 - Classification of histone chaperones in multiple organisms.

chaperones. Histones H3/H4 exist as a dimer when bound to some histone chaperones (Tagami et al., 2004), but they exist as H3/H4 heterotetramers with in the nucleosome. The explanation of this apparent discrepancy was explained by the structure of Asf1

bound to a H3/H4 dimer (English et al., 2006; Natsume et al., 2007), showing that Asf1 physically interacts the H3/H4 tetramerization interphase. The fact that Asf1 binds to newly synthesized dimers of H3/H4 indicates that process of chromatin assembly involves the formation of an H3/H4 heterotetramer from two dimers of H3/H4. This has been still a challenging question to get exact mechanism, whether the H3/H4 heterotetramer is formed on the DNA or prior to being incorporated onto the DNA. Chromatin assembly factor 1 (CAF-1) is a histone chaperone acting in cooperation with Asf1. Recent crystal structures of the p55 subunit of *Drosophila melanogaster* CAF-1 and the related protein human RbAp46 has revealed that they interacted with the alpha helix 1 of histone H4 (Murzina et al., 2008; Song et al., 2008). This interacting region of histone H4 is far from the H3/H4 tetramerization interface, rising the possibility that CAF-1 may be able to bind and deposit tetramers of H3/H4 onto DNA.

1.2.4.1 Histone chaperone N1/N2

N1and N2 was isolated in 1975 as proteins that accumulate in the nucleoplasm when Xenopus nuclear extracts were injected into *Xenopus laevis* eggs (Bonner, 1975; De Robertis et al., 1978). Later, it was shown that N1 and N2 form a complex with histones and possess histone chaperone activity (Kleinschmidt and Franke, 1982). The DNA sequence of *N1* and *N2* genes are identical, and differences in biochemical and immunological properties have not yet been explained. Mammalian NASP (nuclear autoantigenic sperm protein) and yeast Hif1 (Hat1 interacting factor 1) have sequence similarities to N1/N2 (Ai and Parthun, 2004; O'Rand et al., 1992; Poveda et al., 2004; Welch and O'Rand, 1990). N1/N2 and NASP possess two acidic histone-binding clusters in the N-terminal region and a nuclear localization signal in

the C-terminal region (Batova and O'Rand, 1996; Richardson et al., 2000). There are two types of NASP, a testis-specific form (tNASP) and a somatic form (sNASP). This indicated that NASP is involved in the formation of chromosomes in both somatic and sperm cells. Human NASP is present in the histone H3.1 and H3.3 complexes in HeLa cells (Drane et al., 2010; Tagami et al., 2004), indicating its role in the nucleosome assembly. NASP can bind the linker histone H1 and competes with DNA for histone H1 (Alekseev et al., 2003). In addition, NASP interacts with the heat-shock protein HSP90 and activates its ATPase activity (Alekseev et al., 2005). The DNA binding factors E2F and Sp1 bind to the NASP promoter suggesting that they regulate the expression of NASP in S phase (Richardson et al., 2006; Richardson et al., 2001). During the cell cycle, NASP shuttles between the nucleus and cytoplasm. These results indicate that NASP may transport linker histone H1 into the nucleus in the presence of HSP90 in the S phase and deposit this histone onto replicated DNA (Alekseev et al., 2003).

1.2.4.2   Histone chaperone NAP1

Nucleosome assembly protein 1 (Nap1) was originally identified in HeLa cells and is one of few histone chaperones that is capable of recognizing all four core histone proteins (Ishimi et al., 1987). Nap1 is a member of the Nap family of histone chaperone proteins that includes SET/TAF-Iβ, Vps75, as well as the human Nap1 variants Nap1-like proteins 1-5 (Nap1L1-5) and has homologues in yeast, *Drosophila melanogaster* and *Xenopus laevis*. Members of the Nap family of proteins are highly conserved and have a broad range of functions including

transcription activation, assembly and disassembly of nucleosomes, and interaction with other proteins that carry out cellular processes unrelated to chromatin dynamics and transcription activation (Zlatanova et al., 2007). Specifically, Nap1 interacts with cellular proteins that carry out many diverse functions including cell cycle regulation, mediation of nuclear import, control of RNA synthesis and translation, ATPase activity within the mitochondria, lysine biosynthesis, and histone transport and control. In the cell, under physiological conditions, Nap exists as a dimer and does not exist in monomeric form (McBryant and Peersen, 2004). As a chaperone, Nap1 is capable of interacting with both H2A/H2B dimers and H3/H4 tetramers. Generally the ratio of interaction is 1:1, Nap monomer:histone monomer. Specifically, one Nap dimer interacts with one H2A/H2B dimer or two Nap dimers interact with one H3/H4 tetramer (Das et al., 2010; McBryant et al., 2003).

Nap1, have been implicated in maintaining a balance between assembly and disassembly of nucleosomes (Park and Luger, 2006). Previously, Nap1 was known for H2A/H2B dimer removal and exchange (Levchenko and Jackson, 2004; Park et al., 2005). However, evidence suggests a role for Nap1 in disassembly of all four-core histones (Okuwaki et al., 2005). Of significance, the Nap1-dependent disassembly of nucleosomes from the HTLV-1 promoter was the first example of a histone chaperone functioning to disassemble the complete octamer in an acetylation-dependent manner. This reaction occurs in the absence of ATP-dependent chromatin remodeling proteins, transcription initiation, and elongation. This observation was noteworthy as Nap1- mediated disassembly occurs in the absence of ATP, but requires acetyl coenzyme A (acetyl CoA) and the HAT activity of p300 (Sharma and Nyborg, 2008)

1.2.4.3   **Histone chaperone HIRA**

HIRA was first isolated as a protein encoded by a gene within the DiGeorge syndrome critical region of human chromosome 22q11 in 1993 (Halford et al., 1993). HIRA was originally named TUPLE1 because of sequence similarity to the yeast corepressor Tup1 and Drosophila E (sp1). Later it was found that TUPLE1 was more similar to Hir1 and Hir2/Spt1, repressor of histone gene transcription in yeast, and therefore it was renamed the protein HIRA (histone regulatory homolog A) (Lamour et al., 1995). HIRA's primary structure is divided into an N-terminal Hir1-like region and C-terminal Hir2-like region. HIR complex is functionally related to the SWI/SNF complex. The ATP-dependent nucleosome remodeling complex SWI/SNF is required for the expression of the histone genes and is recruited to this locus through its interaction with Hir1 and Hir2 (Dimova et al., 1999). Interactions between HIRA and transcription factors like Pax3 and Pax7 (Magnaghi et al., 1998), functional interactions with transcription elongation factors such as Spt4, 5, 6 and 16 (DeSilva et al., 1998), and Spt phenotype of Hir1 and Hir2 mutants (Sherwood et al., 1993) suggest that HIRA, Hir1 and Hir2 are involved in the regulation of the transcription of several classes of genes. HIRA also interacts with CAF-1 p48 and with HDAC2 through the N-terminal WD40 domain and C-terminal LXXLL motif, respectively (Ahmad et al., 2003).   HIRA is also known to be required for the recruitment of HP1 to pericentromeres in human cells (Zhang et al., 2007). Human HIRA, yeast Hir1 and Hir2 interact with human and yeast CIA respectively. HIRA selectively binds to histone H3.3 and is required for the deposition of histone H3.3 during sperm nucleus decondensation in fertilized *Drosophila eggs* and mouse

zygote (Loppin et al., 2005; Munakata et al., 2000). These results all indicate that HIRA and CIA are coordinately required for replication-independent nucleosome assembly *in vivo*. Structural and functional analyses of the interaction between HIRA and CIA have identified their interaction surface, which are distinct from the sites of interaction between CIA and the histone H3-H4 dimer (English et al., 2006; Natsume et al., 2007). Further structural and functional analyses of HIRA with the CIA-H3-H4 complex will clarify the mechanisms of nucleosome assembly and disassembly.

1.2.4.4   Histone chaperone DAXX

DAXX was initially linked to FAS-mediated apoptosis. DAXX was found to colocalize with both the promyelocytic leukaemia (PML) nuclear body and the alpha-thalassemia/mental retardation X-linked syndrome protein (ATRX), which is highly enriched at pericentric heterochromatin (Lewis et al., 2010). Recently, it is shown that DAXX facilitates the deposition of H3.3 with the help of ATRX (Drane et al., 2010; Santenard et al., 2010). DAXX directly interacts with H3.3 both *in vivo* and *in vitro* and mediates deposition of bacterially purified recombinant H3.3-H4 tetramer and naked DNA in vitro (Santenard et al., 2010).

Interestingly, the DAXX-ATRX complex deposited H3.3 at regions different from the ones that contained H3.3 deposited by HIRA. Genome-wide enrichment studies show HIRA-independent localization of H3.3 at telomeres and transcription factor binding sites (Drane et al., 2010). Surprisingly, CAF1 was found to be associated with H3.3 predeposition complex in the absence of DAXX, suggesting that cells can use replication dependent assembly pathway to counterbalance the loss

of DAXX (Mosammaparast et al., 2002). It seems that DAXX prevents the interaction of H3.3 with CAF-1 complex in order to promote replication independent chromatin assembly of H3.3.

1.2.4.5 Histone chaperone CAF-1

In 1986, Stillman and colleagues identified an activity that preferentially assembles nucleosomes onto replicating DNA (Stillman, 1986), and purified a factor responsible for this activity and named it "chromatin assembly factor-1" (CAF-1) (Smith and Stillman, 1989). Subsequently, CAF-1 homologues were identified in both Drosophila (Kamakaka et al., 1996; Tyler et al., 1996) and yeast (Enomoto et al., 1997). Human CAF-1 (hCAF-1) is composed of three subunits: p150, p60 and p46/p48.

Human p150, *Drosophila* p180, and yeast Cac1/Rlf2 share primary structure homology. The C-terminal region of p150 is involved in a dimer formation and directly interacts with p60, which is essential for CAF-1 activity. Moreover, the N-terminal region of p150 interacts with the DNA polymerase clamp PCNA, and p150 colocalizes at DNA replication foci with PCNA and p60 in the S phase (Krude, 1995; Shibahara and Stillman, 1999). Human p60, which is encoded in the Down's syndrome region of human chromosome 21, possesses HIRA like amino acid sequence (Blouin et al., 1996; Kaufman et al., 1998; Kirov et al., 1998).

Human p60, Drosophila p105, and yeast homologue Cac2 contain a WD40 domain and binds to CIA/Asf1 directly like HIRA (Krawitz et al., 2002; Tang et al., 2006). P60 contains several consensus target sites for various kinases and is phosphorylated by Cyclin/Cdk *in vitro* (Keller and Krude, 2000; Martini et al., 1998).

Active CAF-1 is present in the nucleus during the G1, S, and G2 phases, whereas inactive CAF-1 is present in both nucleus and cytoplasm during the M phase (Marheineke and Krude, 1998). The p60 subunit of CAF-1 is phosphorylated and associates with chromatin in response to UV irradiation, and CAF-1 promotes nucleosome assembly on newly synthesized DNA after UV irradiation. These observations suggest that p60 is a cell cycle and UV-irradiated –dependent regulated subunit of CAF-1.

Human p48, *Drosophila* p55, and *yeast* Cac3/Msi1 contain a WD40 domain like p60. In addition, p48 is present not only in the CAF-1 complex but also in several chromatin-related complexes including HAT1 complex (Martini et al., 1998), the HDAC1 complex (Tyler et al., 1996), the histone methyltransferase complex ESC-E(Z) (Muller et al., 2002), and the ATP-dependent nucleosome remodeling complex NURF (Martinez-Balbas et al., 1998). The p46/p48 subunit of CAF-1 is associated with histone H4 in the absence of other CAF-1 subunits indicating that it links predeposited histones and various chromatin-related complexes (Verreault et al., 1996).

1.2.4.6 Histone chaperone CIA/Asf1

Sternglanz and colleagues isolated anti-silencing function 1 (Asf1) in yeast genetic screen as a factor whose overexpression disrupts both telomeric and mating-type silencing (Le et al., 1997). Asf1 is called CIA (CCG1-interacting factor A) in *S. pombe.* CIA is composed of two structurally distinct domains, an evolutionarily conserved N-terminal core domain and a divergent C-terminal domain. The N-terminal core domain possesses an immunoglobulin fold structure and is sufficient

for the histone chaperone activity. The C-terminal species-specific region of the yeast homologue is rich in acidic amino acid residues (Le et al., 1997; Padmanabhan et al., 2005; Umehara et al., 2002). However, in the human protein Asf1 the C-terminal region is enriched in serine and threonine residues. Although there is only one gene in yeast and *Drosophila* but there are two CIA genes in vertebrates, plants, and *C. elegans*. The vertebrate proteins are CIA-I/Asf1a and CIA-II/Asf1b (Sillje and Nigg, 2001; Umehara and Horikoshi, 2003), which functionally differ in many aspects. (i) HIRA preferentially interacts with CIA-I (Tagami et al., 2004; Tang et al., 2006; Zhang et al., 2005), (ii) CIA-I is more strongly phosphorylated than CIA-II in the C-terminal serine/threonine-abundant regions (Sillje and Nigg, 2001), and (iii) CIA-I is ubiquitously expressed, but CIA-II is expressed in a tissue-specific manner (Umehara and Horikoshi, 2003). In DNA replication, CIA associates with the histone H3.1 chaperone CAF-1 through its p60 subunit in an evolutionarily conserved manner. In addition, the ATPase complex RFC (replication factor C), which loads the DNA polymerase clamp factor PCNA, directly interacts with CIA and recruits CIA to the DNA *in vitro* (Schulz and Tyler, 2006). During the DNA repair, CIA interacts physically and functionally with the FHA domain of RAD53, which is a conserved checkpoint protein kinase (Emili et al., 2001; Hu et al., 2001). These results all show that CIA is involved in various DNA specific reactions including transcription, DNA replication, and DNA repair. CIA is regulated in the cell cycle dependent manner and phosphorylated by S phase specific kinase Tlk in human, *Arabidopsis*, and *Drosophila* cells (Carrera et al., 2003; Ehsan et al., 2004; Sillje and Nigg, 2001). Disruption of CIA results in a growth defect in *S. cerevisiae* and lethality in *S. pombe* (Tyler et al., 1999). In human fibroblast cells, the formation of senescence-associated heterochromatin foci (SAHF) is driven by CIA-I and HIRA (Ye et al., 2007). Histone

chaperones form extensive interaction network and conjunction with other factors participate in a variety of chromatin transactions and cellular task. Recent studies have emphasized a critical role for histone H3/H4 chaperone ASF1 in orchestrating distinct chromatin modifying activities. Asf1 brings histones to CAF1 and HIRA, thus allowing replication-coupled and replication independent chromatin assembly, respectively (Green et al., 2005; Groth et al., 2007a; Tyler et al., 1999; Tyler et al., 2001). The human protein complex CAF-1, composed of three subunits p150, p60 and p48, is the best characterized histone chaperone to promote nucleosome assembly specifically onto newly replicated DNA (Kaufman et al., 1995). CAF-1 also mediates nucleosome assembly coupled to nucleotide excision repair (NER) (Gaillard et al., 1996) and the repair of single-strand breaks (Moggs et al., 2000). Replication-coupled chromatin assembly also involves a complex of ASF1 with MCM2-7 DNA helicase, which is required for DNA unwinding (Groth et al., 2007a). In conjunction with Rtt109 and CBP/p300 acetyltransferase, ASF1 stimulates chromatin assembly and incorporation of H3K56Ac marks at DNA repair foci (Chen et al., 2008; Das et al., 2009). In addition to chromatin assembly, ASF1 cooperates with SWI/SNF family of ATP-dependent chromatin remodelers and H2A/H2B chaperone FACT to displace nucleosomes from active promoters (Gkikopoulos et al., 2009; Takahata et al., 2009). These findings suggest that CIA/Asf1 is involved in a variety of cell functions including cell proliferations, death, and senescence.

1.3 Histone deposition

The first in chromatin assembly is the deposition of H3/H4 onto the DNA, followed by deposition of the two H2A/H2B dimers and later incorporation of linker

histones such as H1. Incorporation of newly synthesized H3/H4 after DNA replication is mediated by the CAF-1, based on their posttranslational modification pattern (Verreault et al., 1996). A role for CAF-1 in chromatin assembly during replication *in vivo* is suggested by the fact that it binds to the replication-specific histone H3.1 variant but not to the replication-independent histone H3.3 variant (Tagami et al., 2004). Interestingly, depletion of human CAF-1 also activates the DNA damage checkpoint and stalls DNA replication itself (Nabatiyan et al., 2006).The histone chaperone Asf1 appears to deliver the newly synthesized histones to CAF-1 for deposition onto the newly replicated DNA. The transfer of newly synthesized histones from Asf1 to CAF-1 is likely to be mediated via physical interaction between these two histone chaperones (Tyler et al., 2001). The role of Asf1 at the replication fork is clearly important because depletion of human, chicken, and Drosophila Asf1 slows down DNA replication (Groth et al., 2005), and slows down DNA unwinding by the MCM helicase in human cells (Groth et al., 2007a). Nucleosome disassembly includes unwinding of the DNA duplex by the MCM2-7 (minichromosome maintenance) helicase complex, followed by passing of the single-stranded DNA through various polymerases. The process of DNA replication is maintained by PCNA (proliferating cell nuclear antigen), a ring like- trimer that encircles the DNA and tethers DNA polymerases to prevent its dissociation. Conceptually, it is difficult to imagine the physical process of threading the DNA through the MCM helicases, PCNA, and DNA polymerases. As such, DNA must be nucleosome free or "naked" while it is replicated. Nearly 250 bp or more of naked DNA resides behind the replication fork (Sogo et al., 1986), while a stretch of approximately 300 bp naked DNA lies ahead of the replication fork (Gasser et al., 1996). Weather this destabilization of nucleosomes ahead of the replication fork is

due to the passage of the replication machinery itself or is mediated by histone chaperones or ATP-dependent chromatin remodelers is not yet known. The loss of the histone octamer from the parental DNA during DNA replication is accompanied by the dissociation of H3/H4 from H2A/H2B. There are candidate histone chaperones for both H2A/H2B and H3/H4 that may disassemble chromatin at the DNA replication fork. However, chromatin assembly and disassembly are so tightly associated with each other during DNA replication that it is very difficult to distinguish between a role for histone chaperone in chromatin assembly and chromatin disassembly. FACT (facilitates chromatin transcription) is an H2A/H2B chaperone during DNA replication. FACT is two subunit complex containing Spt16 (suppressor of Ty) and Pob3 (polymerase one binding) in yeast and SPT16 and SSRP1 (structural specific recognition protein) in humans. FACT is unusual chaperone, which binds with both H2A/H2B and H3/H4 (Stuwe et al., 2008; VanDemark et al., 2008). Human and mouse FACT localize to replication origins (Hertel et al., 1999; Tan et al., 2006), whereas budding yeast FACT interacts with the single-stranded DNA binding protein replication protein A (RPA) (VanDemark et al., 2006), and human FACT interacts with the MCM complex (Tan et al., 2006) The most convincing evidence for a role of Human FACT in chromatin disassembly during DNA replication comes from its ability to promote replication initiation in vivo and to promote DNA unwinding by the MCM helicase on nucleosome templates in vitro (Tan et al., 2006). Another histone chaperone that could be involved in removing H2A/H2B from DNA is Nap1. Nap1 interacts with H2A/H2B and can assemble chromatin *in vitro* (Ito et al., 1996). Nap1 can also disassemble histone octamers from chromatin in concert with the ATP-dependent chromatin remodeling factor RSC *in vitro* (Lorch et al., 2006). However, Interpretation of *in vivo* studies

becomes complicated when the histone chaperones are involved in multiple pathways, when are they essential, and when are they functionally redundant. In nucleosome disassembly steps, it is clear that first H2A/H2B are removed from DNA and then more stable H3/H4 can be removed. One histone chaperone that has potential to remove the H3/H4 from replicating chromatin is Asf1. Asf1 localizes to replication foci in Drosophila S2 cells, but rapidly dissociates when replication is halted in response to hydroxyurea treatment (Schulz and Tyler, 2006). Human Asf1 has been shown to bind to the MCM helicase complex, through histones H3 and H4 and in the absence of Asf1, DNA unwinding by the MCM helicase is greatly reduced *in vivo* (Groth et al., 2007a). To understand the exact mechanism of chromatin disassembly and how the parental pattern of histone variants and histone post-translational modifications are re-established on the daughter strands to ensure epigenetic inheritance, during DNA replication awaits the development of *in vivo* assays for chromatin disassembly during replication.

1.3.1 Replication-independent chromatin assembly

Outside the S phase, histones can be deposited onto DNA by HIRA (HIR complex in *S. cerevisiae* via a replication-independent pathway (Henikoff and Ahmad, 2005). The interaction between Asf1 and Hir was initially found in budding yeast and Asf1 can copurify with all four subunits of HIR (Hir1, Hir2, Hir3 and Hpc2). Similarly, Asf1 forms complexes with histones H3 and H4 as well as HIRA in humans (Figure 7). HIRA preferentially deposits the histone variant H3.3 in nucleosomes (Loppin et al., 2005; Nakayama et al., 2007; Tagami et al., 2004).
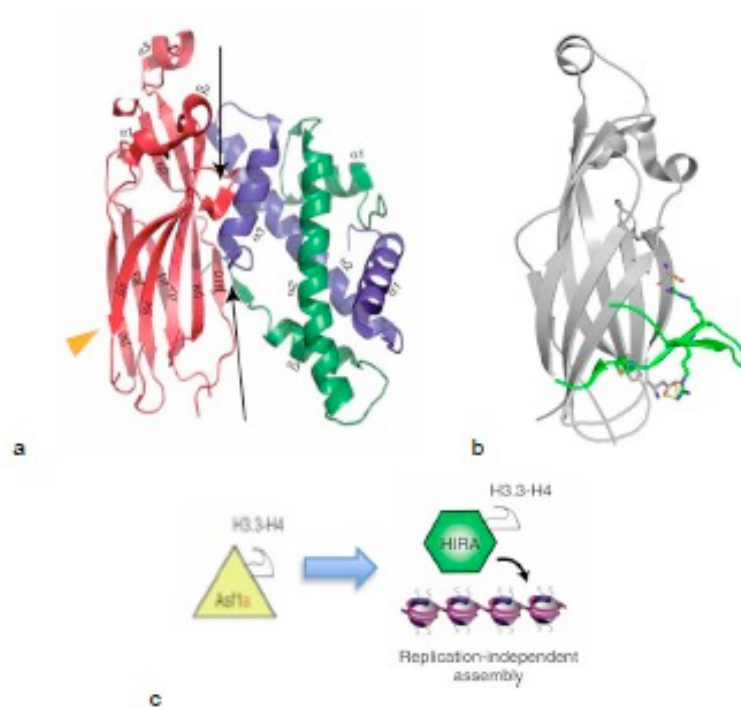
Figure 7: A view of H3.3-H4 deposition by HIRA. (a) The structure of Asf1-H3/H4 complex shows two binding sites for human ASFa on the histone dimer. Orange triangle indicates HIRA-binding site. (b) hAsf1a-HIRA Ribbon diagram of a HIRA B domain peptide (green) bound to hAsf1a N-terminal domain (gray) (Tang et al, 2006). (c) Asf1a cooperates with HIRA to deposit H3.3.

Since H3.3 is predominantly incorporated into actively transcribed genes (Schwartz and Ahmad, 2005; Wirbelauer et al., 2005), the HIRA/ASF1a complex is thought to mediate transcription-coupled deposition of histone H3.3 (Henikoff, 2008; Tagami et al., 2004). The overexpression of HIRA can also inhibit histone expression and lead to an S-phase arrest (Nelson et al., 2002). The human Asf1 N-terminal domain has been shown to interact with the B-domain of HIRA (Daganzo et al., 2003), in the form of an antiparallel β-hairpin (Figure 7b). The evolutionarily

conserved B-domain of HIRA (425-472) is located in the central portion of the protein. This surface is located on the opposite side of the H3 binding site of Asf1 (Tang et al., 2006). The ASF1 D37R+E39R double mutant disrupts the ASF1a-HIRA interaction, but does not affect the ASF1a-H3 complex (Mousson et al., 2005; Tang et al., 2006).

1.3.2 Replication dependent chromatin assembly

The association of DNA with the histones in the nucleosome makes it difficult to access the DNA by protein molecules. The nucleosome is disassembled into two H2A-H2B dimers and a $(H3-H4)_2$ tetramer ahead of the moving fork during DNA replication, transcription and repair. Then, the parental histones are relocated behind the replication fork and the full nucleosome density is completed by the deposition of newly synthesized histones (Falbo and Shen, 2006; Groth et al., 2007a; Tagami et al., 2004). In *S. cerevisiae*, passage through S phase in the absence of core histone synthesis results in a loss of viability that cannot be rescued by re-expression of histones in G2 (Kim et al., 1988). The H2A-H2B chaperone FACT has been shown to be associated with the MCM (Minichromosome maintenance) helicase that unwinds DNA in front of the replication fork (Tan et al., 2006). Asf1 is also associated with the MCM helicase, suggesting that Asf1 plays a role in disrupting parental nucleosomes and potentially transferring them onto the nascent DNA behind the fork (Groth et al., 2007a). Asf1 acts in both chromatin assembly and disassembly (Adkins et al., 2004; Adkins et al., 2007b; Korber et al., 2006). A complex of Asf1 in association with the histones H3 and H4 was purified from *Drosophila melanogaster* embryonic extracts as a factor that could assemble nucleosomes onto replicating

DNA in a synergistic manner with CAF-I (Tyler et al., 1999). CAF-I is itself a H3/H4 histone chaperone composed of three subunits that was unified from HeLa cell nuclear extract as a factor that could assemble nucleosomes onto the SV40 DNA *in vitro* (Smith and Stillman, 1989). These biochemical results suggest that Asf1 and CAF-1 cooperate in nucleosome assembly onto newly replicated DNA during the S-phase and Asf1 binds to p60 subunit of CAF-1 in yeast, Drosophila, chicken, and human (Krawitz et al., 2002; Mello et al., 2002; Sanematsu et al., 2006; Tang et al., 2006). The way these two histone chaperones cooperate in the nucleosome assembly coupled to DNA replication is still a matter of debate. CAF-1 and Asf1 co-purified in a soluble predeposition complex together with the replication-specific histone H3.1 in HeLa cells (Tagami et al., 2004). CAF-1 is recruited to the replication fork through interaction with cell proliferating nuclear antigen (PCNA) and PCNA is loaded onto DNA by replication factor C (RFC) (Majka and Burgers, 2004; Shibahara and Stillman, 1999). Thus, Asf1 and CAF-I could both be associated with the progressing replication fork and cooperate to deposit H3/H4 onto DNA. Yeast Asf1 and both human isoforms Asf1a and Asf1b can interact with CAF-1 p60, promoting replication-dependent chromatin assembly synergistically with CAF-1 (Figure 8) (Loyola and Almouzni, 2004; Mello et al., 2002; Sharp et al., 2001; Verreault, 2000).
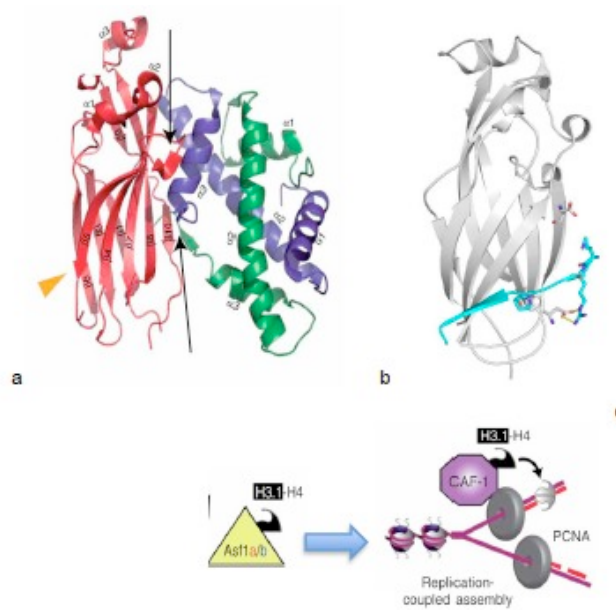
Figure 8. A view of H3.1-H4 deposition by CAF-1. (a) The structure of Asf1-H3/H4 complex shows two binding sites for human ASFa on the histone dimer. Orange triangle indicates CAF-1-binding site. (b) Interaction of yeast Asf1 (SpAsf1) with a peptide from the p60 subunit of CAF-1, spCac2 (Malay et al, 2008). (c) Both Asf1a and Asf1b act as histone donors for CAF-1, promoting H3.1 deposition (De Koning et al., 2007).

This pathway ensures that histones are promptly assembled onto newly replicated DNA to minimize the potential for DNA damage, as well as being important for the inheritance of the epigenetic information during DNA replication and repair (Groth et al., 2007a; Henikoff et al., 2004). CAF-1 associates with the replication forks through an interaction with proliferating cell nuclear antigen (PCNA), a component essential for DNA replication and DNA repair (Shibahara and Stillman, 1999; Zhang et al., 2000). CAF-1 is essential in humans, as depletion of p60CAF-1 triggers apoptosis in proliferating cells (Nabatiyan and Krude, 2004). In

contrast to human cells, Asf1 and CAF-1 are not essential for cell viability in *S.cerevisiae*, probably because of the existence of other chaperones for histone H3-H4, such as Rtt106 (Huang et al., 2005). Besides Asf1, CAF-1 has been shown to mediate histone deposition onto DNA, assisted by Rtt106 that binds to CAF-1 as well (Huang et al., 2005). Although CAF-I is not essential in *S. cerevisiae*, its inactivation results in increased sensitivity to UV radiation and reduced silencing of genes adjacent to telomeric DNA (Kaufman et al., 1997).

1.4 Dynamic roles of the histone chaperone Asf1

1.4.1 Structure of Asf1

The Asf1 gene is found at one or two copies per haploid genome in all examined eukaryotes. Asf1a/CIA-I is the major isoform expressed in mammals and appears to be widely expressed, whereas Asf1b/CIA-II shows restricted tissue specific distribution with high-level expression in the testis and thymus, and small level in the intestine and colon (Umehara and Horikoshi, 2003). Both isoforms are similar in sequence (around 70% identical amino acids). In all species, Asf1 consists of a highly conserved 155-amino acid N-terminal domain and a variable C-terminal tail. In human Asf1a, the N-terminal domain contains 23 acidic residues vs only 9 basic ones. Indeed few acidic positions are strictly conserved and are involved in the binding of H3 and HirA (Daganzo et al., 2003; Mousson et al., 2005). Non-conserved residues and the variable C-terminal tail might be involved in species-specific regulation of binding interactions. It is noteworthy that the C-terminal tail of Asf1a,

and to a lesser extent Asf1b, is very rich in serine and threonines, which are the targets of Tousled-like kinases (Sillje and Nigg, 2001). 3-D structure of the functional N-terminal domain of budding yeast Asf1 and human Asf1a were resolved by X-ray crystallography and nuclear magnetic resonance (NMR), respectively. NMR experiments performed on the intact hAsf1a protein revealed that its C-terminal tail is unstructured and highly flexible (Mousson et al., 2005; Padmanabhan et al., 2005). The N-terminal domain containing 155 residues is the highly-conserved core region of Asf1. In contrast, the C-terminal tail is variable, unstructured and flexible (Daganzo et al, 2003; Mousson et al, 2005). In *S. cerevisiae* and *S. pombe,* the Asf1 C-terminal sequence is extremely rich in asparatates and glutamates and this type of tail is common in histone chaperones. In vertebrates, the Asf1 C-terminal sequences are not as rich in acidic residues, but they are phosphorylated by Tousled-like kinases (Tlks). The major Tlks phosphorylation sites are located in the C-terminal part of *Drosophila melanogaster* and human Asf1 within a (D/E)-N-S-(L/M) consensus motif, and both proteins cooperate in control of chromatin dynamics and cell cycle progression. The loss of Tlk activity or mutation of phosphorylation sites of Asf1 results in degradation of Asf1 by both proteasome-dependent and independent pathways (Pilyugin et al., 2009). The N-terminal domain of Asf1 consists of three helical linkers on top of a compact immunoglobulin-like β-sandwich fold. This domain is sufficient for all currently known functions of the full-length protein. Asf1 has a large electro-negative surface potential surrounding one side, and a highly conserved hydrophobic groove that interacts with histone. The 3D structure of the functional N-terminal domain of budding yeast was determined by X-ray crystallography (Figure 9)(Daganzo et al., 2003).
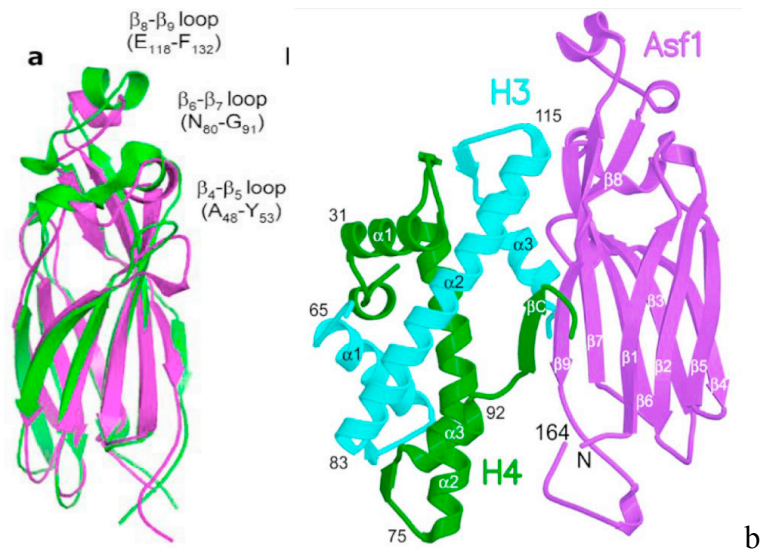
Figure 9: Structure of the Asf1 N-terminal domain is well conserved. The superposition between hAsf1a (1-156) (purple) and *S. cerevisiae* Asf1(green) (Daganzo et al., 2003; Mousson et al., 2005). (b) Ribbon diagram model of the Asf1p N-terminal domain bound to heterodimer histone H3 (cyano) and H4 (green) (English et al, 2006).

Florence Mousson and Françoise Ochsenbein determined the structure of the human Asf1a N-terminal domain by nuclear magnetic resonance spectroscopy (NMR) (Mousson et al., 2005). The structures of yeast and human Asf1 N-terminal domains are quite similar.

1.4.2 Asf1-H3/H4 complex structure

Structure shows that Asf1 binds to a histone H3/H4 heterodimer. The hydrophobic groove of Asf1 binds the histone H3-H4 heterodimer by enveloping the

C-terminus helix of histone H3, thereby blocking the formation of a $(H3-H4)_2$ heterotetramer (English et al., 2006). Furthermore, the C-terminus of histone H4, that forms a mini-β sheet with histone H2A in the nucleosome, undergoes a major conformational change upon binding to Asf1 and adds a β strand to the Asf1 β sheet sandwich. Additionally, the non-conserved acidic C-terminal tail of yeast Asf1 may strengthen the interaction between Asf1 and H3/H4 (English et al., 2006; Natsume et al., 2007).

1.4.3 Asf1 in transcriptional regulation

In yeast, Asf1 facilitates chromatin disassembly at the PHO5 promoter to promote transcriptional activation, suggesting that it acts as a histone acceptor (Adkins and Tyler, 2004). Asf1 travels with the transcription machinery and/or rapidly fills in gaps left in nucleosome arrays following passage of RNA polymerase (Schwabish and Struhl, 2006). The nature of Asf1 as an interactor with the TFIID subunit Bdf1 also suggests its participation in transcription control at various RNA polymerase II-dependent gene loci (Zabaronick and Tyler, 2005). Asf1 is also involved in developmental gene expression control by mediating transcriptional repression of NOTCH target genes in *Drosophila melanogaster* (Goodfellow et al., 2007). In yeast, the loss of Asf1 results in impaired cell proliferation and minor defects of gene silencing at telomere and silent mating loci HMR and HML. These effects are greatly enhanced by inactivation of CAF-1, but not by Hir. Asf1 and Hir participate together in a pathway for telomeric silencing that is independent of a pathway dependent on CAF-I (Daganzo et al., 2003). In addition, Asf1 was found to mediate histone H3 eviction and deposition during transcriptional elongation.

Furthermore, Asf1 has been implicated in transcription dependent, but replication-independent histone H3 exchange at promoters, another process which can deposit K56-acetylated H3.

1.4.4 Asf1 in DNA damage response

Asf1 has been strongly implicated in the cellular response to DNA damage. In budding yeast, Asf1 is bound to the Rad53 DNA damage checkpoint kinase during normal growth but this association is abolished when yeast cells are subjected to genotoxic stress (Emili et al., 2001). Upon genotoxic stress, RAD53 is activated by its phosphorylation on multiple sites through the action of the upstream Mec1 and Tel1 kinases, as well as Rad53 autophosphorylation (Gilbert et al., 2001; Sanchez et al., 1996). This multiple phosphorylated form of Rad53 is not found to be associated with Asf1. Several models have been proposed to explain how Asf1 and Rad51 function in response to DNA damage. The interaction may serve to recruit the Asf1-Rad53 complex to sites of DNA damage. Phosphorylation and activation of Rad53 at these sites would release Asf1 for binding histone H3/H4 and other potential partners.

1.4.5 Asf1 in histone modifications

Asf1 can affect the PTM state of histones. Asf1 also contributes to the acetylation of H3K9 and can promote trimethylation of H3K36 by Set2 in yeast

(Adkins et al., 2007a). In human U2OS cells, histones bound to Asf1 showed two typical chromatin marks: H4K16Ac and H3K9Me3, giving rise to the hypothesis that Asf1 handles both new and parental histones during DNA replication. Asf1 and H3K56Ac Acetylation of H3K56 is an abundant modification of all newly synthesized H3 in budding yeast, fission yeasts and in Tetrahymena thermophiles, but is much less abundant in mammalian cells (Groth et al., 2007a; Jasencakova et al., 2010). H3K56 is located at the DNA entry/exit point on the nucleosome core. Although H3K56 acetylation does not appear to greatly alter the overall structure of the nucleosome, acetylation at this residue increases the plasticity of nucleosomes, which may facilitate access of necessary protein factors to the DNA. Asf1 physically interacts with Rtt109 and is absolutely required for H3K56 acetylation. H3K56 acetylation is required for S-phase chromatin assembly and was proposed to be a critical signal for turning off the DNA damage checkpoint following DNA but this claim is still controversial (Chen et al., 2008). H3K56 acetylation can increase the binding affinity between H3-H4 with CAF-1 and Rtt106 to promote efficient deposition of H3-H4 onto replicating DNA by these two histone chaperones. Asf1 is thought to maintain the integrity of the replisome through H3K56 acetylation. Asf1 has been shown to directly interact with origins of replications and can also associate with components of the replisome (Groth et al., 2007b). It appears that Asf1 and H3K56 acetylation promote the stability of stalled replications forks, contributing to cellular survival upon replication stress.

2 Aim of the study

The presented study addresses the elementary processes that govern the establishment of chromatin structure and its dynamic properties. Since chromatin acts as a key element that controls access to the DNA, the assembly and altering of its structure are of central importance for cellular function. The main factors that determine chromatin assembly, reconfiguration and dynamics are histone chaperones and chromatin remodeling complexes. Here, two of these factors, the chaperone Asf1a and the Asf1b class-remodeling complex were investigated in HeLa cells. Key questions that were addressed include the molecular mechanisms for the biological activity of both factors and their relation to the assembly and dynamics of chromatin in replication independent (RI) and replication coupled (RC) manner.

Manuscript

An extended Hinge domain of heterochromatin protein 1 discriminates between Asf1a and Asf1b to constitute specific H3-H4 deposition complexes

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Abbreviations: Asf1, (anti-silencing function 1); CAF-1, chromatin assembly factor 1; HP1, heterochromatin protein 1

Keywords: Asf1, CAF-1, chromo domain (CD), chromoshadow domain (CSD), Hinge domain (HD), HP1, histone deposition, nucleosome assembly

60

Abstract

Asf1 is a highly conserved histone chaperone that facilitates histone deposition and removal during nucleosome assembly and disassembly. Asf1 cooperates with the chromatin assembly factor 1 (CAF-1) to promote replication-dependent chromatin assembly and with HIRA to promote replication-independent chromatin assembly. Mammalian cells possess two isoforms of Asf1; Asf1a and Asf1b. However to date, whether they have individual functional roles has remained elusive. Here, we purified the epitope tagged Asf1a and Asf1b complexes from HeLa cells soluble nuclear extracts and analyzed their specific interacting partners. Mass spectrometry analysis followed by immunoblotting revealed that Asf1a associates exclusively with HP1⟨ and Asf1b with HP1®. The selectivity of this interaction can be recapitulated *in vitro* using highly purified recombinant proteins. Mutational analysis revealed that the two Asf1 isoforms discriminate between HP1⟨ and HP1® through their divergent C-terminal tail, which selectively recognizes an extended non-conserved Hinge domain encompassing the first 11 amino-acids of either HP1⟨ or HP1® chromoshadow domains. The interaction of Asf1 with the Hinge domain has important functional implications and might serve to counteract the binding of non-coding RNA to HP1. Our data elucidate for the first time the functional importance of Asf1 isoforms and reveal specific interactions with the different HP1 isoforms. Asf1a/b may associate with specific HP1 isoforms to promote nucleosome occupancy at defined chromatin loci.

Introduction

Histone chaperones play essential roles in chromatin dynamics during the different steps of gene transcription, DNA repair and replication. Histone chaperones are responsible for delivery of histones to the site of chromatin assembly and histone deposition onto DNA, histone exchange and removal (Shuaib et al., 2010). The histone chaperone Asf1 (anti-silencing function 1) is important for S-phase progression (Sanematsu et al., 2006; Tyler et al., 1999) and was first identified by its capacity to derepress silencing when overexpressed in yeast (Le et al., 1997). Structural and biochemical studies show that Asf1 binds only to one H3–H4 dimer (English et al., 2006; English et al., 2005; Natsume et al., 2007) thus preventing the formation of H3–H4 tetramer. The role of Asf1 is to provide H3.1-H4 dimers to CAF-1 for replication-dependent nucleosome assembly (Mello et al., 2002; Tyler et al., 1999) and to HIRA to promote replication-independent chromatin assembly (Galvani et al., 2008; Green et al., 2005; Tagami et al., 2004). Asf1 binds histone H3-H4 heterodimers in the cytoplasm and together are imported to the nucleus with thanks to the association with importin-4 with subsequent transfert to downstream chromatin assembly factors (Alvarez et al., 2011; Campos et al., 2010; De Koning et al., 2007; Jasencakova et al., 2010). Asf1 binds canonical S-phase histones H3.1-H4 as well as replacement histones H3.3-H4, which are transferred to CAF-1 and HIRA, respectively (Green et al., 2005; Tagami et al., 2004). However, the precise mechanism by which histones H3.1-H4 and H3.3-H4 are delivered to chromatin are not yet fully understood. For instance, it is not yet known whether Asf1 needs additional factor to fulfill the deposition reaction. However, it is known that

the CAF-1 via its p150 subunit, requires to interact with heterochromatin protein 1 (HP1) for pericentric heterochromatin replication and S-phase progression (Murzina et al., 1999; Quivy et al., 2008).

In yeast, only one form of Asf1 has been identified, in contrast to mammalian cells, which possess two isoforms of Asf1; Asf1a and Asf1b. Although, both isoforms have redundant functions in S-phase histone dynamics, Asf1b appears to be more specific to proliferating cells (Corpet et al., 2011) suggesting that the two isoforms may exert different intimate functions. Asf1a and Asf1b are highly homologous but only Asf1a has been shown to be part of the HIRA-containing complex (Tagami et al., 2004; Zhang et al., 2005) and directly interacts with a central B-domain of HIRA (Tang et al., 2006). Asf1a belongs to a large multicomponent complex containing Ubinuclein 1 (UBN1) and the Calcineurin-binding protein 1 (CABIN1) that is involved in HIRA-dependent H3.3 deposition (Elsaesser and Allis, 2010; Tagami et al., 2004). Therefore, Asf1a appears to be associated to replication-independent nucleosome assembly and thus underlying a possible role outside DNA replication such as gene silencing of proliferating-promoting genes (Galvani et al., 2008; Gazin et al., 2007).

Asf1b is rather specific for proliferating cells and it has been suggested that it acts as the prominent histone acceptor/donor during DNA replication (Corpet et al., 2011). Depleted Asf1b cells show increased nucleus morphology abnormalities, which have been suggested to result from replication defects during mitosis (Corpet et al., 2011; Galvani et al., 2008). Asf1b is a 202 amino-acid long, *i.e.*, 2 amino-acids shorter than Asf1a, but it differs from the latter by 58 amino-acids, most of them being located in the C-terminal part of the protein, suggesting that if there is a difference in functions between both proteins, it will come from the C-terminal part. In spite of this difference, no difference in the partners of Asf1a and Asf1b could be found, which

were NASP, codanin, importin, Ku70, p60CAF-1, p48/p46CAF-1 and HAT1 (Kang et al., 2011). Exceptions are Ubp11, an ubiquitin protease, and S100A8 (a member of the S100 family), that are found to be associated with Asf1b but not with Asf1a (Kang et al., 2011). Recently, data from the literature show some evidence that Asf1 may associate with HP1 to promote nucleosome occupancy at heterochromatic loci (Yamane et al., 2011).

Mammalian cells have three isoforms of HP1 that fulfill different functions: HP1〈, HP1® and HP1© (Kwon and Workman, 2008). HP1〈 and HP1® preferentially localize to heterochromatin whereas HP1© is found in euchromatin and functions in gene-specific silencing (Ayyanathan et al., 2003). HP1® can also be found in euchromatic regions. Indeed, HP1b (*Drosophila* homologue) plays important roles in transcriptional activation and development. It has been suggested that HP1b may counteract HP1a function both in heterochromatin formation
and in the transcriptional regulation of euchromatic genes (Kwon and Workman, 2008). HP1〈 appears to be as an active factor in early DNA damage signaling and repair pathways (Baldeyron et al., 2011). HP1® explores chromatin for sites of methyl-mark enrichment where it can bind H3 tails from adjacent nucleosomes (Munari et al., 2012).

In search for further, specific partners for each Asf1 isoform, we purified the epitope tagged-Asf1a and the epitope tagged-Asf1b complexes by using the double-immunoaffinity purification method. Among the Asf1a and Asf1b partners we could confirm the already known proteins that co-purified, NASP, HAT1, importin-4 and p150CAF-1. Surprisingly, but highly interesting and relevant, was the discovery that heterochromatin protein 1 alpha (HP1〈) associates with the Asf1a complex and heterochromatin protein 1 beta (HP1®) with the Asf1b complex. Furthermore, we observed that each isoform of Asf1 physically interacts with its respective HP1

isoform. We mapped the domain of HP1⟨ and Asf1a that are involved in this interaction and called them "Extended Hinge" (EH) and HP1 Interacting Domain (HID), respectively. We propose that the interaction between EH and HID domains governs specific roles for Asf1 and HP1 isoforms in the deposition of histone H3-H4 dimers in a spatio-temporal dependent process.

Results

Isolation of the Asf1a and Asf1b specific nuclear soluble complexes and identification of specific partners

We used the double-immunoaffinity purification method (Drane et al., 2010; Ouararhni et al., 2006) to isolate the epitope tagged Asf1a and Asf1b specific nuclear soluble complexes (e-Asf1a.com, e-Asf1b.com) from HeLa cells. Epitope tagged Asf1a (e-Asf1a) and Asf1b (e-Asf1b) proteins were stably expressed as fusion protein with N-terminal Flag- and HA-epitope-tags in Hela cells. The epitope tag HA allowed us first to investigate the nuclear localization of each protein. Asf1a expression pattern shows a specific staining throughout the nucleoplasm, forming numerous dots whereas Asf1b shows a more diffuse staining throughout the nucleus (Fig. 1A). Epitope-tagged Asf1a and Asf1b deposition complexes were then purified by double immunoaffinity from HeLa soluble nuclear extracts by sequential immunoprecipitations with anti-Flag antibody followed by anti-HA antibody (Drane et al., 2010; Ouararhni et al., 2006). Proteins associated with Asf1a and Asf1b were separated by SDS-containing 4%-12% polyacrylamide gradient gels and subsequently silver-stained (Fig.1B). Mass spectrometry and immunoblotting analyses allowed the identification of RIF1, MCM2-7, tNASP, sNASP,

MMS22L, NFKBIL2, HAT1, TLK1/2, Codanin-1, RbAp46 (RBBP7), RbAp48 (RBBP4), histones H4 and H3 as components of the Asf1a and Asf1b complexes (Figs. 1B, 1C & 1D). RIF1, Rap1-interacting factor 1 homolog, a critical determinant of the replication-timing program (Cornacchia et al., 2012; Yamazaki et al., 2012). MCM2-7 is the replicative helicase. tNASP and sNASP are two isoforms of NASP, an H1 histone-binding protein that is cell cycle-regulated. tNASP is found in gametes, embryonic cells, and transformed cells whereas sNASP is found in all rapidly dividing somatic cells (Richardson et al., 2000). MMS22L and NFKBIL2 proteins, which are part of the MMS22L-TONSL complex required to maintain genome integrity during DNA replication by promoting homologous recombination-mediated repair of replication fork-associated double-strand breaks (Duro et al., 2010; O'Connell et al., 2010; O'Donnell et al., 2010). HAT1 is a histone acteyltransferase that has been recently been reported to preferentially acetylate histone H4 in H3.1-H4 dimer over H3.3-H4 dimer. TLK1/2, serine/threonine-protein kinase tousled-like 1/2, involved in Asf1 phosphorylation (Sillje and Nigg, 2001). Codanin-1, an Asf1 interacting protein regulating S-phase histone supply (Ask et al., 2012). Other proteins have also been identified with very good scores, but surprisingly were more enriched in the Asf1a complex than in the Asf1b complex, such as the HIRA complex (HIRA, Cabin1, Ubinuclein 1 and 2) and the CAF-1 complex (p60CAF-1 and p150CAF-1) (Fig. 1D). To our surprise, we found HP1⟨ in the e-Asf1a complex and HP1® in the e-Asf1b complex, suggesting a specific role for each couple in the histone H3/H4 deposition probably depending on a spatio-temporal process.

Asf1a and Asf1b physically interacts with HP1⟨ and HP1®, respectively

Considering that we found the same proteins in each complex, although some of them in different amounts, we hypothesized that each isoform of Asf1 is able to discriminate between the HP1 isoforms through a direct interaction. To address this question, GST-Asf1a or GST-Asf1b fusions together with His-HP1⟨ or His-HP1® or His-HP1© were coexpressed in bacterial cells. Then, GST-Asf1a or GST-Asf1b, together with their associated proteins, were purified at high salt concentration (1M NaCl) and run on a SDS gel and the gel was stained with colloidal blue. The data obtained clearly show that HP1⟨ binds stoichiometrically to Asf1a (Fig. 2A) but not to Asf1b (Fig. 2B). Conversely, HP1® does not bind to Asf1a (Fig. 2C) but binds stoichiometrically to Asf1b (Fig. 2D). Asf1a exhibited weak binding to HP1© (Fig. 2E) in contrast to Asf1b, which showed strong binding to HP1 © (Fig. 2F). This result is consistent with the sequence alignment of HP1 isoforms (Fig. S1A), showing that HP1© is more closely related to HP1® than to HP1⟨, particularly regarding the Hinge domain.

To map the domain of interaction between Asf1a and HP1 ⟨, we generated various deletion mutants of HP1⟨ and Asf1a. HP1⟨ (191 amino-acid long) is constituted of a chromodomain (17-71), a Hinge domain (86-108) and a chromoshadow domain (116-176) (Fig. 3A). We constructed five deletion mutants for HP1⟨, ⊗1(72-191), ⊗2(1-115), ⊗3(116-191), ⊗4(72-176) and ⊗5(86-126) (Fig. 3A). These mutants were co-expressed with Asf1a in bacteria and investigated for their binding to the GST-Asf1a fusion protein by a GST pull-down assay. The HP1⟨ mutant ⊗1(72-191) was found to be able to bind to Asf1a (Fig. 3B, ⊗1) but neither the ⊗2(1-116) (Fig. 3B) nor the ⊗3(116-191) (Fig. 3B) could bind. Interestingly, when the Hinge domain was present but supplemented with and extension at least up to amino-acid126, overlapping the beginning of the chromoshadow of HP1, the mutants ⊗4(72-176) and ⊗5(86-

126) exhibited binding to Asf1a (Figs. 3B, ⊗4 and 3B, ⊗5). These results show that the Hinge domain of HP1⟨ is not sufficient for binding to Asf1a but requires an additional extension up to amino-acid 126, which we call "Extended Hinge" (EH). This finding is consistent with the sequence alignment of the three isoforms of HP1, which show that major difference in the primary sequences lies in the extended Hinge domain (Fig. S1A). Therefore, we suggest that the Hinge domain HP1⟨ has the capacity to discriminate between the Asf1 isoforms. We propose that such mechanism also occurs for the HP1®/Asf1b.

Next, we wanted to map the Asf1a domain involved in the interaction with HP1⟨. For this, we assumed that the most likely domain is located in the C-terminal part of Asf1a considering that it holds the major difference in the primary sequences of both isoforms. We constructed two deletion mutants of Asf1a namely ⊗1(1-160) and ⊗2(161-204) (Fig. 3F) fused with GST to perform GST pull-down assays. The mutant ⊗1(72-191) lacking the 44 amino-acids of the C-terminal part of Asf1a was unable to bind to HP1⟨. In contrast, the construct containing the 44 amino-acid fused with the GST exhibits interaction capacity with HP1⟨ showing that it is the C-terminal part of Asf1 that is involved in the interaction. We named this domain of Asf1a the "HP1 Interaction Domain" or "HID" (Fig. S1B).

Reconstitution of the Asf1a, HP1⟨, CAF-1 and H3/H4 deposition complex

We next investigated how Asf1a and HP1⟨ are integrated in the CAF-1 complex and assayed to stepwise reconstitute the Asf1a/HP1⟨/CAF-1/H3-H4 deposition complex. We first investigated the ability of Asf1a to bind to the H3.1/H4 dimer and to the H3.3-H4 dimer *in vitro* using a GST pull-down assay. GST-Asf1a was co-expressed in bacteria with H3.1 and H4 or

H3.3 and H4. Glutathione bead-bound proteins were eluted and fractioned on SDS-PAGE and stained with colloidal blue. Asf1a strongly bound to either H3.1-H4 or H3.3-H4 dimers and this interaction resisted to 1M NaCl (Fig. 4A and 4B). When co-expressing additionally HP1⟨, we observed that this latter associates with Asf1a, H4 and either with H3.1 (Fig. 4C) or with H3.3 (Fig. 4D). This result shows that HP1⟨ is a part of the nucleosome assembly complex and is probably involved in an early stage of this process.

The identification of the CAF-1 subunit that interacts with Asf1a and Asf1b, respectively, was the next step. CAF-1 is composed of three subunits, *i.e,* p150, p60 and p48/46. p150CAF-1 and p60CAF-1 were highly specific to the e-H3.1 complex, whereas p46/48 was a component common to e-H3.1 and e-H3.3 complexes (Drane et al., 2010; Tagami et al., 2004). Asf1 is the histone chaperone that is donating new histones to CAF-1. We have assessed the interaction of the three CAF-1 subunits with each Asf1 isoform by using a GST pull-down assays. We have produced and purified GST-Asf1a and GST-Asf1b fusion proteins from bacteria and incubated them with the three different CAF-1 subunits produced in baculovirus (Figs. 5A and 5B). Inputs of GST-Asf1a/b (lane 2), p150 (lane 3), p60 (lane 5) and p48 (lane 7) are shown (Fig. 5A and 5B). Asf1a or Asf1b did not interact with p150 (lane 4) and p48 (lane 8) but did interact with p60 (lane 6). These results show that Asf1a and Asf1b are both able to interact with CAF-1 by binding to the p60 subunit.

Our further challenge was to reconstitute the Asf1a, HP1⟨, CAF-1, H3.1-H4 complex in the presence of HP1⟨ to check whether this latter is a component of the histone predeposition complex. For this, we incubated the three CAF-1 subunits in the presence of GST-Asf1a, HP1⟨, H3.1 and H4 and performed a GST pull-down assay. Glutathione bead-bound proteins were eluted and fractioned on SDS-PAGE and stained with colloidal blue (Fig. 5C). The data obtained

show that HP1⟨ associates with the histone predeposition complex, *i.e,* with H3.1, H4, CAF-1 and Asf1a. Our results suggest that HP1⟨ is associated with Asf1a before being recruited to chromatin.

Discussion

In search for specific partners for each Asf1 isoform, we purified the epitope tagged Asf1a and Asf1b complexes from HeLa cells soluble nuclear extracts and analyzed their specific interacting partners. Our data revealed an unexpected association of Asf1a and Asf1b with the specific HP1 isoforms. Asf1a associates exclusively with HP1⟨ and Asf1b with HP1®. The selectivity of these interactions have important biological implications and could explain how Asf1a/b throughout their association with specific HP1 isoforms could help establishing specialized chromatin structures.

The genome of many examined eucaryotes, with the exception of yeast and fungi, contains two Asf1-encoding genes (Kawai et al., 2001; Munakata et al., 2000). The Asf1 homologues are highly conserved throughout evolution (80% identity) with similar structure; while the carboxyl-termini are very diverse among species (Daganzo et al., 2003; Mousson et al., 2005). The high similarity between Asf1 isoforms causes difficulties in relating functional diversities to sequence variations. The purification and analysis of the two Asf1 isoforms from human cells allowed us to characterize in details their interacting partners. Among the partners, we find some proteins that have already been described as part of the Asf1 complex, *e.g.,* HIRA, HAT1, NASP, MCM2-7, Codanin-1, importin, and CAF-1. No significant differences in the composition of the two complexes could be observed except for one protein, which was HP1 of

70

which a different isoform was found in each complex. Consistently with this result, it is known

that CAF-1 is a partner of Asf1 and of HP1 (Eitoku et al., 2008) thus showing the relevancy of

the observation that Asf1 and HP1 are found in the same complex. Highly interesting and novel,

we found that HP1⟨ associates with the Asf1a complex and HP1® associates with the Asf1b

complex. Considering, that so far the only differences of the Asf1a complex and Asf1b complex

are HP1⟨ and HP1 ®, we hypothesized that each isoform of Asf1 is able to discriminate between

the two HP1 isoforms through a putative direct interaction. That is what was investigated and

observed *in vitro* by using GST pull down assays. Indeed, we observed that Asf1a through its

non-conserved carboxyl-termini directly binds to HP1⟨ whereas Asf1b binds to HP1®. The

selectivity of each binding is highlighted by a complete absence of binding of Asf1a to HP1® and

vice versa. We next asked whether the third HP1 isoform, HP1©, could discriminates between

the two Asf1 isoforms. Interestingly, HP1© was found to be able to bind to both Asf1 isoforms

with some preference for Asf1b. This result is not surprising given that HP1© is more closely

related to HP1® than to HP1⟨ (Fig. S1A).

All HP1 proteins share the same architecture consisting of two structured and conserved

domains, a chromodomain (CD) and a chromoshadow domain (CSD), connected by a less

conserved Hinge domain (Jacobs and Khorasanizadeh, 2002; Zeng et al., 2010; Fig. S1A). We

next, characterized the interaction of Asf1a with the different HP1⟨ domains and identified the

specific Asf1 interaction motif. This domain extends from the beginning of the HP1⟨ Hinge

domain up to the first 11 amino-acids of the chromoshadow. As a consequence, we called it

"Extended Hinge". To our knowledge, it is the first time that a protein was reported to interact

with the Hinge domain of HP1⟨. So far only RNA has been reported to directly recognize the

Hinge domain. Indeed, it was found that the Hinge domain of HP1α strongly recognized both forward and reverse centromeric RNA probes, whereas the chromo-hinge and the hinge-chromoshadow domains showed lower binding capacities (Maison et al., 2011). Previously, it was suggested that the RNA binding activity of the Hinge domain would be implicated in the targeting of HP1α to chromatin (Muchardt et al., 2002). In *Xenopus laevis,* it has been reported that it is the Hinge domain that is responsible for targeting HP1 to native chromatin (Meehan et al., 2003). Interestingly, the Hinge domain is the site where HP1α can be sumoylated in order to be targeted to pericentromeric chromatin (Maison et al., 2011). This would suggest that sumoylation might be a potent regulatory process for the interaction between Asf1a and HP1α. In the light of our results, we can speculate that HP1 is recruited to chromatin via an Asf1-CAF-1-PCNA mediated chromatin assembly pathway. Our reconstitution experiments of the recombinant CAF-1 complex support this view and suggest that Asf1 is integrated in the CAF-1 complex through its association with p60CAF-1 subunit (Fig. 5). CAF-1 is recruited to replication foci by PCNA through an interaction with p150CAF-1 subunit (Shibahara and Stillman, 1999). We do not exclude that sumoylation would stabilize the Asf1 and HP1α interaction and thus facilitates the recruitment of HP1α to chromatin via PCNA.

We do not yet know the precise significance of the association of Asf1a with HP1α and Asf1b with HP1β. Consistently, with our present data, showing no mixing of HP1α and HP1β in the soluble complexes of Asf1 isoforms, it has been previously shown that HP1α targeting causes the recruitment of endogenous HP1β to the chromatin region and *vice versa* (Verschure et al.,
2005). The isoforms of HP1 are all homodimers thanks to the chromoshadow domain, in which each monomer, the chromodomain is binding the H3K9me2 or H3K9me3. HP1α appears to be

as an active factor in early DNA damage signaling and repair pathways (Baldeyron et al., 2011). Considering, that Asf1a appears to be associated to replication-independent nucleosome assembly (Gazin et al., 2007), it is plausible that the Asf1a complex containing HP1⟨, would be rather involved in DNA damage repair process. It is known that only HP1® is essential for cell viability (Singh, 2010). Thus, we suggest that the Asf1a complex is dispensable for cell viability but indispensable for chromatin repair processes.

It has long been considered that HP1 is stably bound to H3K9me3 epigenetic mark without dynamic exchange, but recent findings have shown that HP1 is highly mobile and forms a stable compartment that is actively renewed (Ayoub et al., 2008; Cheutin et al., 2003; Festenstein et al., 2003). We propose that the renewal of HP1 is governed by the dynamical behavior of the Asf1 complexes and we anticipate that HP1 sumoylation might play an important role in this dynamic. We also suggest that the highly flexible nature of HP1 in its free and bound forms plays an important role in its fundamental biological activity to connect different nuclear processes, as it enables the two other HP1 binding platforms, *i.e.,* the chromo domain and the chromoshadow domain, to be adapted within the dense chromatin architecture to recruit additional proteins.

Given the direct connection between Asf1a/b and HP1⟨/®, it will be interesting to address the role of Asf1 isoforms in tissue specification at different developmental stages. Mouse Asf1a shows a ubiquitous expression pattern, whereas Asf1b is highly expressed in the testis and thymus, with little expression in the small intestine and colon (Umehara and Horikoshi, 2003). These results support the view that the two Asf1 isoforms have different functions. Our results reporting differential interactions between Asf1a/b and HP1⟨/® provide a conceptual framework for understanding the different functions of Asf1a/b in the context of chromatin.

Acknowledgments

Materials and Methods

Cell lines

Human Asf1a and Asf1b proteins fused with N-terminal Flag-, HA- and His-epitope tags (e-Asf1a and e-Asf1b) were stably expressed in HeLa cells by retroviral transduction (Ouararhni et al., 2006).

Plasmid construction

Human Asf1a and Asf1b cDNAs were PCR amplified and cloned into the XhoI-NotI sites of pGEX-5X1 (Amersham) to generate pGEX-5X1-Asf1a (1-204) and pGEX-5X1-Asf1b (1-202) constructs. The deletion mutants pGEX-5X1-Asf1a (1-161) and pGEX-5X1-Asf1a (162-204) were obtained by cloning the PCR products amplified from pGEX-5X1-Asf1a (1-204) into the XhoI-NotI sites of the vector.

Human HP1⟨, HP1®, HP1© cDNAs were PCR amplified from a human cDNA library and cloned into the XhoI-NotI sites of pET28b generating pET28b-HP1⟨, pET28b-HP1® and pET28b-HP1© full length constructs. The deletion mutants pET28b-HP1⟨ (1-115), pET28b-HP1⟨ (72-191), pET28b-HP1⟨ (116-191), pET28b-HP1⟨ (72-176), pET28b-HP1⟨ (86-126) and

pET28b-HP1⟨ (86-115) were obtained by cloning the PCR products amplified from pET28b-HP1⟨ into the XhoI-NotI sites of the pET28b vector. The deletion mutant pET28b-HP1® (86-115) was obtained by cloning the PCR product amplified from pET28b-HP1® into the XhoI-NotI

sites of the pET28b vector.

Double-Immunoaffinity purification

To identify the proteins interacting with Asf1a and Asf1b, we established stable HeLa cell lines expressing a N-terminal FLAG-HA-His epitope tagged Asf1a and Asf1b (e-Asf1a and e-Asf1b). Extracts were prepared using a modification of the Dignam protocol (Dignam, 1990). Briefly, cells were lysed in hypotonic buffer (10 mM Tris-HCl at pH 7.65, 1.5 mM $MgCl_2$, 10 mM KCl) and disrupted by Dounce homogenizer. The cytosolic fraction was separated from the pellet by centrifugation at 4°C. The nuclear-soluble fraction was obtained by incubation of the pellet in high-salt buffer in order to get a final NaCl concentration of 300 mM. Tagged proteins were immunoprecipitated with anti-Flag M2-agarose (Sigma), eluted with Flag peptide (0.5 mg/mL), further affinity-purified with anti-HA antibody-conjugated agarose, and eluted with HA peptide (1 mg/mL). The HA and Flag peptides were first buffered with 50 mM Tris-Cl (pH 8.5), then diluted to 4 mg/mL in TGEN 150 buffer (20 mM Tris at pH 7.65, 150 mM NaCl, 3 mM $MgCl_2$, 0.1 mM EDTA, 10% glycerol, 0.01% NP40), and stored at −20°C until use. The proteins associated with e-Asf1a and e-Asf1b were separated on 4-12% gradient PAGE containing SDS and silver stained using the Silver Quest kit (Invitrogen). Identification of proteins was carried out using an Orbitrap mass spectrometer (ThermoFinnigan Orbitrap LTQ-Velos) or by Taplin Biological Mass Spectrometry Facility (Harvard Medical School, Boston, MA).

Western blotting and antibodies

Western blotting was carried out using standard protocol and antibodies employed were as follows: monoclonal antibody anti-Flag M2 (Sigma); pan anti-H3 Ct (05-928, Upstate Biotechnologies); polyclonal anti-NASP (ProteinTech Group); anti-MCM (BD Pharmingen); anti-HAT1 (ProteinTech); anti-HP1⟨ and anti-HP1® was produced by the IGBMC facility.

Immunofluorescence

Immunofluorescence was performed using standard procedures on a Leica DMR microscope (Leica) using a 63 x 1.32 NA oil immersion objective. Rat anti-HA antibody (Roche) was used at 1:400 dilution; the secondary antibody used is a goat anti-rat IgG coupled to Alexa Fluor 488 (Molecular Probes) at 1:400 dilution.

Expression and purification of proteins

Appropriate plasmids were transformed in *Escherichia coli* strain BL21-CodonPlus-RIL-pLysS (Stratagene) and induced with 0.5 mM IPTG at 30°C for 3 h. Cells were harvested by centrifugation and processed for protein purification. GST-fusion Asf1a, Asf1b and its deletion mutants were purified as a GST-tagged fusion protein by glutathione-sepharose affinity chromatography (GE healthcare). HP1⟨, HP1®, HP1© and their deletion mutants containing His-tag was purified using Ni-NTA agarose resin according to the kit protocol (QIAGEN).

Human His-p48CAF-1, FLAG-p60CAF-1 and HA-p150-CAF-1 were cloned in the pFast-bac1 vector and produced in the baculovirus system according to the manufacturer's recommendation (Invitrogen). Recombinant proteins were purified by the respective affinity

chromatography from the cell lysates using standard protocols in TGN 500 buffer (20 mM Tris at pH 7.65, 500 mM NaCl, 3 mM MgCl$_2$, 10% glycerol, 0.01% NP40). Purified proteins were gel quantified using BSA as a standard and stored at −80°C until use.

Protein–protein interactions

GST fusion proteins immobilized on glutathione Sepharose were incubated for 2 h at room temperature with recombinant proteins in TGEN buffer (20 mM Tris at pH 7.65, 3 mM MgCl$_2$, 0.1 mM EDTA, 10% glycerol, 0.01% NP40) containing 250 mM NaCl. Beads were then washed extensively in TGEN containing 250 mM, 500 mM, or 1 M NaCl. Bound proteins were eluted in SDS sample buffer and fractionated on SDS-PAGE.

To study the interaction between GST-Asf1a and GST-Asf1b with HP1 isoforms, GST-tag constructs were coexpressed with his-tagged HP1 constructs in *Escherichia coli* strain BL21-CodonPlus-RIL-pLysS (Stratagene), as described in (Shuaib et al., 2010).

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Figure legends

Figure 1

Purification and characterization of e-Asf1a and e-Asf1b soluble nuclear complexes from HeLa cells. (*A*) Stable expression of e-Asf1a and e-Asf1b in HeLa cells. Cells expressing e-

Asf1a and e-Asf1b and control cells (CTRL) were stained with anti-HA (*right*) and DAPI (*left*). (*B*) Silver staining of proteins associated with e-Asf1a (lane *3*) and e-Asf1b (lane *4*) complexes. The complexes containing e-Asf1a (e-Asf1a.com) and e-Asf1b (e-Asf1b.com) were purified by double immunoaffinity from soluble nuclear extracts. Mock, indicate mock purification from non-tagged HeLa cells (*lane 2*) Polypeptides identified by mass spectrometry analysis and the positions of molecular size markers are indicated. (*C*) Hp1⟨ and Hp1® proteins are respectively specific to the e-Asf1a and e-Asf1b complexes. The e-Asf1a (lane *3*) and e-Asf1b (lane *4*) complexes were analyzed by immunoblotting with the indicated antibodies. (*D*) mass spectrometry table indicating proteins identified in the e-Asf1a and e-Asf1b complexes.

Figure 2

Selective interactions of Asf1a and Asf1b with the different Hp1 isoforms. (A) Asf1a physically associates with Hp1⟨ when co-expressed in bacteria. Bacterial cells were either non-induced (NI) or induced (NI) with 0.5 mM IPTG to co-express GST-Asf1a and His-Hp1⟨ (lanes 2 and 3). The Asf1a/Hp1⟨ complex was purified and washed with 1M NaCl (lane 4). The purified material was separated on SDS-PAGE and stained with Colloidal blue. M, indicates the molecular weight marker. (B) Asf1b does not associate with Hp1⟨ when co-expressed in bacteria. Bacterial cells were either non-induced (NI) or induced (I) with 0.5 mM IPTG to co-express GST-Asf1b and His-Hp1⟨ (lanes 2 and 3). The GST-pulled down material was washed with 1 M NaCl, eluted and separated on SDS-PAGE. Different concentrations of Asf1b were loaded on the gel to ascertain the absence of Hp1⟨ in the pull-down (lanes 4-6). M, indicates the

molecular weight marker. (C) Asf1a does not associate with Hp1® when co-expressed in bacteria. Bacterial cells were either non-induced (NI) or induced (I) with 0.5 mM IPTG to co-express GST-Asf1a and His-Hp1® (lane 2 and 3). The GST-pulled down material was washed with 1 M NaCl, eluted and separated on SDS-PAGE (lane 4). M, indicates the molecular weight marker. (D) Asf1b physically associates with Hp1® when co-expressed in bacteria. Bacterial cells were either non-induced (NI) or induced (NI) with 0.5 mM IPTG to co-express GST-Asf1b and His-Hp1® (lanes 2 and 3). The Asf1b/Hp1® complex was purified and washed with 1M NaCl (lane 4). The purified material was separated on SDS-PAGE and stained with Coloidal blue. M, indicates the molecular weight marker. (E) Asf1a weakly associates with Hp1© when co-expressed in bacteria. Bacterial cells were either non-induced (NI) or induced (NI) with 0.5 mM IPTG to co-express GST-Asf1a and His-Hp1© (lanes 2 and 3). The Asf1a/Hp1© complex was purified and washed with 1M NaCl (lane 4). The purified material was separated on SDS- PAGE and stained with Colloidal blue. M, indicates the molecular weight marker. (E) Asf1b strongly associates with Hp1© when co-expressed in bacteria. Bacterial cells were either non- induced (NI) or induced (NI) with 0.5 mM IPTG to co-express GST-Asf1b and His-Hp1© (lane 1 and 2). The Asf1b/Hp1© complex was purified and washed with 1M NaCl (lane 3). The purified material was separated on SDS-PAGE and stained with Colloidal blue.

Figure 3

The distal C-terminal tail of Asf1a interacts with the Extended Hinge domain of Hp1⟨. (**A**) Schematics of Hp1⟨ domain organization and mapping. Hp1⟨ contains three different domains: the N-terminal Chromo domain (CD), the central Hinge Domain (HD) and the C-terminal chromoshadow domain (CSD). (**B**) Interaction of Asf1a with Hp1⟨ deletion mutants.

Bacterial cells were either non-induced (NI) or induced (NI) with 0.5 mM IPTG to co-express full-length GST-Asf1a with His-Hp1⟨ deletion mutants (Δ1–Δ5). The purified bound material (lane B) was washed with 1M NaCl, eluted, separated on a SDS-PAGE and stained with colloidal blue. M, indicates the molecular weight marker. (C) Schematics of Asf1a domain mapping. (D) Hp1⟨ interacts with the distal C-terminal tail of Asf1a. Interaction of Hp1⟨ with Asf1a deletion mutants. Bacterial cells were either non-induced (NI) or induced (NI) with 0.5 mM IPTG to co-express full-length His-Hp1⟨ with GST-Asf1a deletion mutants (Δ1 and Δ2). The purified bound material (lane B) was washed with 1M NaCl, eluted, separated on a SDS- PAGE and stained with colloidal blue. M, indicates the molecular weight marker.

Figure 4

Reconstitution of the four Asf1a subunits complex. (A) Asf1a strongly interacts with H3.1. Full-length GST-Asf1a, together with H3.1-H4 histones, were coexpressed and purified from bacteria. The purified material was washed with different salt concentrations, eluted, separated on a SDS-PAGE and stained with colloidal blue. (B) Asf1a strongly interacts with H3.3. Full-length GST-Asf1a, together with H3.3-H4 histones, were coexpressed and purified from bacteria. The purified material was washed with different salt concentrations, eluted, separated on a SDS-PAGE and stained with colloidal blue. (C) Reconstitution of the Asf1a, HP1⟨ and H3.1-H4 deposition complexe. Full-length GST-Asf1a, together with Hp1⟨ and H3.1-H4 histones, were coexpressed and purified from bacteria. The purified bound material (bound) was washed with 1M NaCl, eluted, separated on a SDS-PAGE and stained with colloidal blue. M, indicates the molecular weight marker. (D) Reconstitution of the Asf1a, HP1⟨ and H3.3-H4

deposition complexe. Full-length GST-Asf1a, together with Hp1⟨ and H3.3-H4 histones, were coexpressed and purified from bacteria. The purified bound material (bound) was washed with 1M NaCl, eluted, separated on a SDS-PAGE and stained with colloidal blue.

Figure 5

Reconstitution of the CAF-1 complex. (A and B) Identification of the CAF-1 subunit interacting with Asf1a and Asf1b. Recombinant GST-Asf1a or GST-Asf1b were incubated with either one of the CAF-1 subunits (p48, p60 and p150). The bound material was purified by GST-pull down, separated on a SDS-PAGE and stained with colloidal blue. M, indicates the molecular weight marker. (B) Reconstitution of the seven CAF-1 subunits complex. The human CAF-1 subunits (p48, p60 and p150) were reconstituted by overexpression of the three subunits in Sf9 cells, mixed with bacterially purified GST-Asf1a four subunits complex (Asf1a, HP1⟨ and H3.1-H4) and followed by GST purification to reconstitute the seven CAF-1 subunits complex.

Figure S1

(A) Multiple primary sequence alignments of the human Hp1 isoforms. **(B)** Multiple primary sequence alignments of the human Asf1a and Asf1b isoforms.

Figure 1

A

DAPI   HA

e-Asf1a

e-Asf1b

CTRL

B

M   Mock   Asf1a.com   Asf1b.com

kD

200–

116–
97–

66–

55–

36–

31–

21–

14–

1   2   3   4

Asf1a.com: CAF150, MCM2-7, sNASP, RbAp46, RbAp48, HAT1, e-Asf1a, HP1α, H3, H4

Asf1b.com: CAF150, MCM2-7, sNASP, RbAp46, RbAp48, HAT1, e-Asf1b, HP1β, H3, H4

C

Asf1a.com   Asf1b.com

MCM2-7
tNASP
sNASP
HP1α
HP1β
HAT1
H3
e-Asf1a
e-Asf1b

D

Proteins	Asf1a.com peptides	Asf1b.com peptides	Accession #	
RIF1	106	46	Q5UIP0	
HP1	6	0	Q6I9T7	Hp1s
HP1	0	5	P83916	Hp1s
Asf1a	6	0	Q9Y294	Histone chaperones
Asf1b	0	7	Q9NVP2	Histone chaperones
NASP	45	38	P49321	Histone chaperones
CHAF1A	18	0	Q13111	CAF-1.com
CHAF1B	24	0	Q13112	CAF-1.com
RBBP4	9	11	Q09028	CAF-1.com
RBBP7	10	7	Q16576	CAF-1.com
HIRA	25	2	P54198	HIRA.com
UBN1	7	0	Q9NPG3	HIRA.com
UBN2	13	7	Q6ZU65	HIRA.com
CABIN1	19	4	Q6ZU66	HIRA.com
MMS22L	25	15	Q6ZU67	MMS22L-TONSL.com
NFKBIL2	10	11	Q6ZU68	MMS22L-TONSL.com
MCM2	61	48	Q6ZU69	MCM2-7.com
MCM3	33	11	Q6ZU70	MCM2-7.com
MCM4	62	41	Q6ZU71	MCM2-7.com
MCM5	26	3	Q6ZU72	MCM2-7.com
MCM6	50	36	Q6ZU73	MCM2-7.com
MCM7	30	12	Q6ZU74	MCM2-7.com
TLK1	28	24	Q6ZU75	Tousled-like kinases
TLK2	26	25	Q6ZU76	Tousled-like kinases
HAT1	21	18	Q6ZU77	
Codanin-1	24	24	Q6ZU78	
Importin-4	42	40	Q6ZU79	

Figure 2

Figure 3

A

B

C

D

Figure 4

Figure 5

Fig. S1A

```
                10        20        30        40        50        60
                 |         |         |         |         |         |
Hp1_beta    ----------MGKKQNKKKVEEVLEEEEEYVVEKVLDRRVVKGKVEYLLKWKGFSDEDN
Hp1_gamma   MASNKTTLQKMGKKQNGK-SKKVEEAEPEEFVVEKVLDRRVVNGKVEYFLKWKGFTDADN
Hp1_alpha   ----------MGKKTKRT-ADSSSSEDEEEYVVEKVLDRRVVKGQVEYLLKWKGFSEEHN
                     ****  :.   ..   . : **:***********:*:***:******::  .*
                                              ---------------CD-------------

                70        80        90       100       110       120
                 |         |         |         |         |         |
Hp1_beta     TWEPEENLDCPDLIAEFLQSQKTAHETDK------SEGGKRKADSDSEDKGEESKPKKKK
Hp1_gamma    TWEPEENLDCPELIEAFLNSQKAGKEKD---------GTKRKSLSDSES--DDSKSKKKR
Hp1_alpha    TWEPEKNLDCPELISEFMKKYKKMKEGENNKPREKSESNKRKSNFSNSA--DDIKSKKKR
             *****:*****:**   *::. *  :* :        . ***:  ...   :: *.***:
             ------------    CD -----------    ------------Hinge-------------

               130       140       150       160       170       180
                 |         |         |         |         |         |
Hp1_beta     EES-EKPRGFARGLEPERIIGATDSSGELMFLMKWKNSDEADLVPAKEANVKCPQVVISF
Hp1_gamma    DAA-DKPRGFARGLDPERIIGATDSSGELMFLMKWKDSDEADLVLAKEANMKCPQIVIAF
Hp1_alpha    EQSNDIARGFERGLEPEKIIGATDSCGDLMFLMKWKDTDEADLVLAKEANVKCPQIVIAF
             : : :  .*** ***:**:*******.*:*******::*****  *****:****:**:*
                    -----------------------     CSD------------------

               190       200
                 |         |
Hp1_beta     YEERLTWHSYPSEDDDKKDDKN---
Hp1_gamma    YEERLTWHSCP-EDEAQ--------
Hp1_alpha    YEERLTWHAYP-EDAENKEKETAKS
             ********: * **   :
             -----CSD-----
```

Fig. S1B

```
                10        20        30        40        50        60
                 |         |         |         |         |         |
Asf1a     MAKVQVNNVVVLDNPSPFYNPFQFEITFECIEDLSEDLEWKIIYVGSAESEEYDQVLDSV
Asf1b     MAKVSVLNVAVLENPSPFHSPFRFEISFECSEALADDLEWKIIYVGSAESEEFDQILDSV
          ****.* **.**:*****:.**:***:***  * *:::**************:**:****
          --------------------H3 binding domain----------------------

                70        80        90       100       110       120
                 |         |         |         |         |         |
Asf1a     LVGPVPAGRHMFVFQADAPNPGLIPDADAVGVTVVLITCTYRGQEFIRVGYYVNNEYTET
Asf1b     LVGPVPAGRHMFVFQADAPNPSLIPETDAVGVTVVLITCTYHGQEFIRVGYYVNNEYLNP
          *********************.***::***********:*************** *:.
          --------------------H3 binding domain----------------------

               130       140       150       160       170       180
                 |         |         |         |         |         |
Asf1a     ELRENPPVKPDFSKLQRNILASNPRVTRFHINWEDNTEKLEDAESSNPNLQSLLSTDALP
Asf1b     ELRENPPMKPDFSQLQRNILASNPRVTRFHINWDNNMDRLEAIETQDPSLGCGLPLNCTP
          *******:*****:*******************::*  ::**  *:.:*.* . *. :. *
          --------H3 binding domain-----------    ---Hp1 binding domain--

               190       200
                 |         |
Asf1a     SASKGWSTSENSLNVMLESHMDCM
Asf1b     --IKGLGLPGCIPGLLPENSMDCI
            ** . .    .:: *. ***:
          ---Hp1 binding doman
```

DISCUSSION

AND

FUTURE PERSPETIVES

Role of the Interaction of Asf1 with HP1: where does it occur?

Asf1 is a highly conserved histone chaperone that facilitates histone deposition and removal during nucleosome assembly and disassembly. Histones can be deposited into chromatin either in a replication-dependent manner or in a replication-independent manner. Interestingly, Asf1 is involved in both ways of histone deposition pathways but not well characterized. Asf1 cooperates with the chromatin assembly factor 1 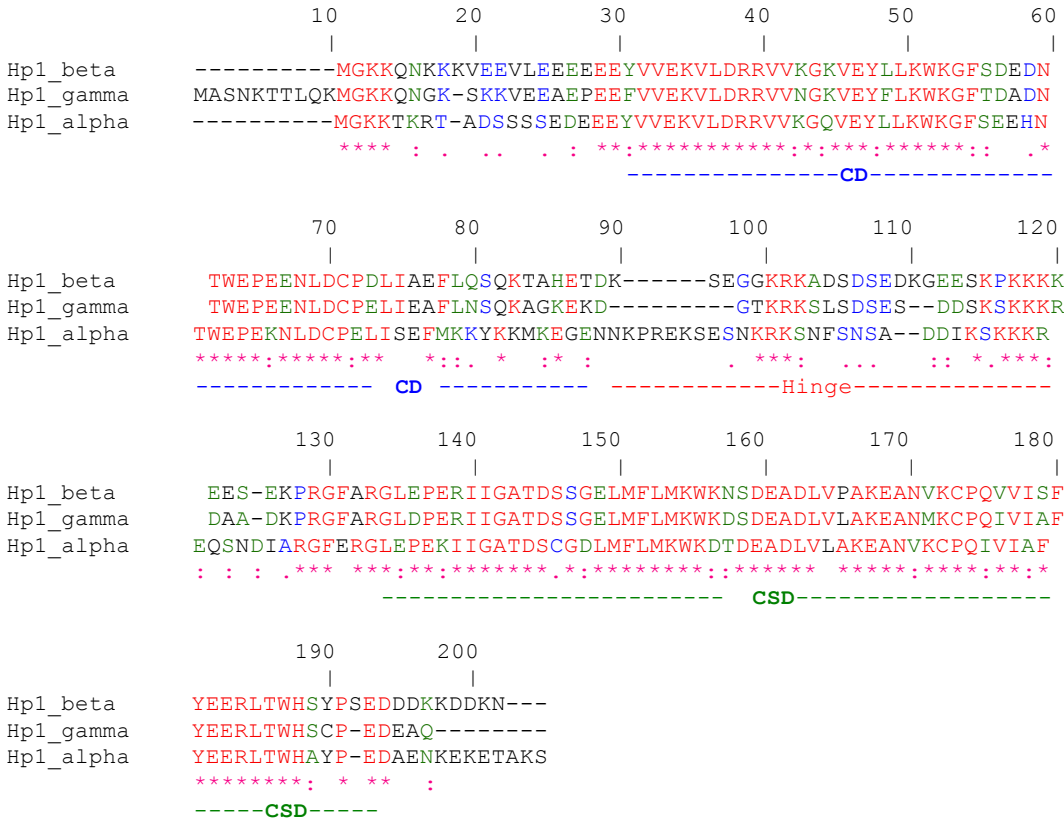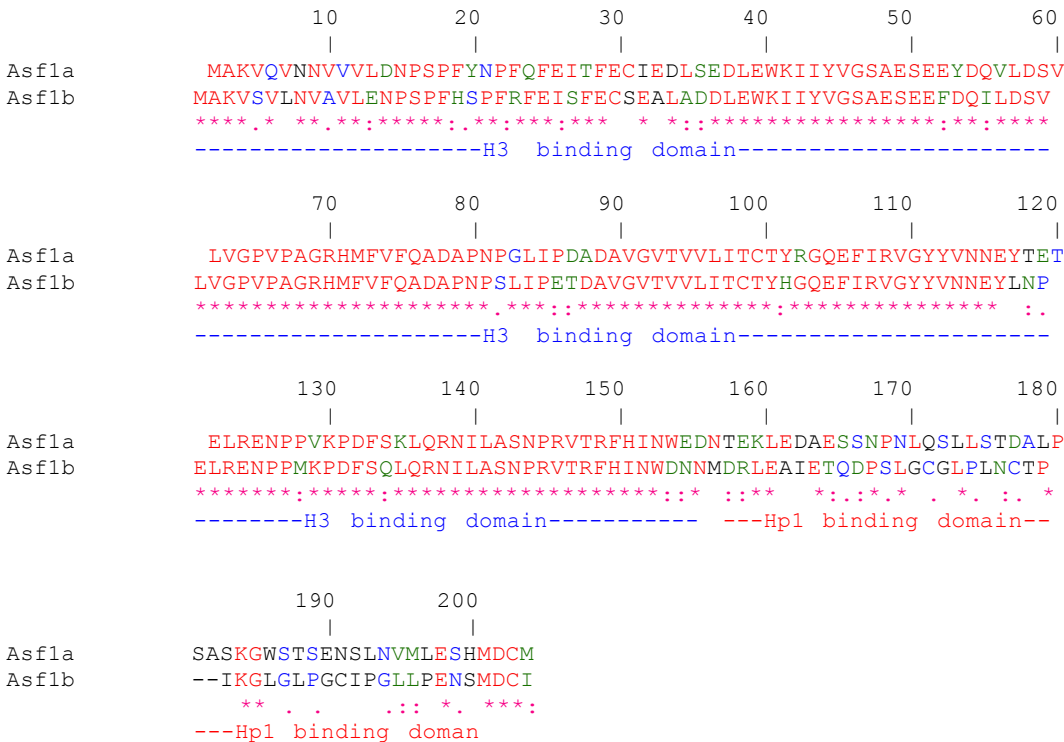(CAF-1) to promote replication-dependent chromatin assembly and with HIRA to promote replication-independent chromatin assembly. In the nucleus the newly synthesized H3–H4 dimers appeared to remain initially associated with Asf1. Next Asf1 supplies the newly synthesized H3–H4 dimers to CAF-1. The p150 subunit of CAF-1 is recruited to the site of DNA synthesis through direct interaction with proliferating cell nuclear antigen (PCNA) and colocalizes with the replication foci and p60 during S-phase (Moggs et al., 2000). Importantly, CAF-1 was found associated *in vivo* with the replication dependent H3.1 complex and not with the replication independent H3.3 complex, a key finding further demonstrating the direct implication of CAF-1 in replication coupled deposition (Tagami et al., 2004). Asf1also supplies newly synthesized H3.3-H4 dimers to HIRA for histone deposition in a replication-independent manner. This deposition occurs at gene bodies (Goldberg et al., 2010).

In our thesis work, we could demonstrate that the histone chaperone Asf1a specifically and directly interacts with HP1a and conversely Asf1b specifically and also directly interacts with HPb. Such specific interaction has never been reported in the literature. This novel and intriguing interaction push us to go deeper in the investigations by identifying the interacting domains on both proteins. All HP1 isoforms exhibit the same characteristic domain organization, but differ in their functions, expression profiles and chromosomal localization. Noteworthy, we could identify a new functional domain in HP1a, that we called "Extended Hinge" (ED). This domain was found to interact with the C-terminal domain of Asf1, which we called

"Heterochromatin protein 1 Interacting Domain" (HID). To our knowledge, it is the first time that a partner of protein nature has been reported to interact with the Hinge domain of HP1a. So far only RNA has been reported to directly recognize the Hinge domain of HP1. Indeed, it was found that the Hinge domain of HP1a strongly recognized both forward and reverse centromeric RNA probes, whereas the chromo-hinge and the hinge-chromoshadow domains showed lower binding capacities (Maison et al., 2011). Previously, it was suggested that the RNA binding activity of the Hinge domain would be implicated in the targeting of Hp1a to chromatin (Muchardt et al., 2002). In *Xenopus laevis,* it has been reported that it is the Hinge domain that is responsible for targeting Hp1 to native chromatin (Meehan et al., 2003). According to our results, one might imagine that the Hinge domain facilitates HP1a to be targeted to chromatin via an interaction with HID domain of Asf1a and probably Asf1b facilitates HP1b to be recruited by the chromatin. Altogether, these results support a key regulatory role for the Hinge domain of HP1 in the regulation of gene transcription and genome integrity.

Asf1a, but not Asf1b has been shown to be a part of the HIRA-containing complex (Tagami et al., 2004; Zhang et al., 2005). This would mean that Asf1b in association with HP1b is never found in HIRA complexes. However, it is known that HP1b co-localises with HIRA complexes in human cells (Zhang et al., 2007). Our results show that Asf1b is found in HIRA complexes, by demonstrating that HIRA is enriched in the Asf1a complex *versus* the Asf1b complex. HIRA mediates H3.3 deposition at pericentromeric regions to ensure, with the help of HP1b, kinetochore structure integrity (Zhang et al., 2007). We suggest that HP1a can also be involved in the maintenance of kinetochore structure integrity considering that we found HIRA and HP1a in the Asf1a complex. Although, HP1a and HP1b localize together at heterochromatic loci, microscopy analysis showed that both isoforms do not exactly overlap (Dialynas et al., 2007) suggesting that they do not associate and that they may have different functions in the pericentromeric chromatin. We propose that these different functions may be set by

Asf1 isoforms, which discriminates between HP1 isoforms during the constitution of the Asf1 complexes.

Concerning HP1g, we could show that HP1g has a preference for Asf1b. HP1g is found in euchromatin and function in gene-specific silencing (Ayyanathan et al., 2003). Our results suggest that HP1g may be brought to euchromatin by the Asf1b complex. It has been observed that HP1g plays an unexpected role in the activation of gene transcription that is dependent on its association with RNA polymerase II (Vakoc et al., 2005). Therefore, our results support the idea that it is the Asf1b complex that might associate with RNA polymerase II in euchromatin to ensure transcription activation of specific genes. However, this requires further investigations.

Interaction of Asf1 with HP1: when does it occur?

Unfortunately, so far we have not yet deciphered the role of each isoform specific interaction in the histone deposition and transcription regulation during the cell cycle, which would have helped us to understand specific histone deposition mechanisms. Nevertheless, we will discuss now when the Asf1/HP1 association might occur.

The first step in the deposition of the newly synthesized histones is the transport from the cytoplasm to the nucleus, a process, which is assisted by distinct chaperones. The chaperone Asf1 was the first chaperone identified to play a key role in supplying histones H3–H4 to the downstream chaperones, like CAF-1 and HIRA for nucleosome assembly. Structural and biochemical studies show that Asf1 binds only to one H3–H4 dimer, thus preventing the formation of H3–H4 tetramer. In addition, the cytosolic complex of ectopically expressed epitope-tagged H3.1 (e-H3.1) contains only the tagged H3.1 fusion, but not the endogenous H3. These data evidence that Asf1 is associated with a single H3–H4 dimer both *in vitro* and *in vivo* and that the H3–H4 dimer, and not the $(H3–H4)_2$ tetramer, is further delivered to the nucleus. In two very recent studies, the

biochemical purification of the cytoplasmic H3 complex has allowed both the identification of distinct H3 chaperones and the suggestion of a comprehensive mechanism(s) for the sequential assembly of H3–H4 dimers. The first study, carried out by the group of D. Reinberg, identified six different H3 chaperones (HCS70, HSP90, tNASP, sNASP, RbAp46, and Asf1b) along with histone H4, importin4 and HAT1. This study has not detected Asf1a as a part of the cytosolic H3 complex. Four distinct cytosolic complexes were found to be sequentially involved in the assembly of H3–H4 dimers (Campos et al., 2010). After synthesis, histones H3 and H4 were sheltered from misfolding and aggregation by interaction with chaperones HSC70 and HSP90, respectively. For transport and deposition onto DNA, histones H3–H4 first assembled to form the dimer, a process facilitated by HSP90 and tNASP. Once the H3–H4 dimer was assembled, RbAp46 associates with the H4 carboxyl domain and helps the recruitment of HAT1, which in turn acetylates H4 on Lys5 and Lys12. Then the acetylated histones are transferred to Asf1b and importin4 for nuclear transport (Campos et al., 2010). The other study, carried out by the group of A. Loyola, reported some different results. First, they described an interesting finding concerning the free soluble H3–H4 dimers. Loyola and colleagues showed that the free soluble endogenous histones H3 and H4 were transiently poly-ADPribosylated and that this mark was removed after dimer formation (Alvarez et al., 2011). Moreover, the two distinct translocation complexes contained H3–H4 dimers with distinct pre-deposition marks. The first translocation complex contained Asf1a, H3K9me1, and H3K14ac, while the second complex contained Asf1b and H3K9me1. This study also claimed that both H3 acetylation and methylation do not affect nuclear translocation, but may be instead linked to the nucleosomal histone H3 PTM patterns (Alvarez et al., 2011). Thus, it is possible that HP1a as well as HP1b may associate with the each isoform of Asf1 before nuclear import.

It has been recently observed that Asf1a accumulated inside the nucleoli of proliferating C7 and C8 cells (Jiang et al., 2011). This accumulation was suggested to be

associated with cell growth and the release of Asf1a from nucleoli with growth arrest. Accordingly, we might propose that Asf1a is playing such a role with the help of HP1a. In contrast, Asf1b had much less nucleolar localization than Asf1a. Asf1a is involved in both DNA replication-dependent and DNA replication-independent nucleosome assembly processes whereas Asf1b is only involved in DNA replication-dependent (Tagami et al., 2004; Tang et al., 2006; Zhang et al., 2005; Mello et al., 2002). As a consequence, considering our results showing that each Asf1 isoform interacts with a different HP1 isoform, we propose that HP1a would be involved in both DNA replication-dependent and DNA replication-independent, while HP1b would only be involved in DNA replication-dependent.

Concluding remarks

It has been recently questioned about the factors that allow each isoform of HP1 to ensure a specific function in spite of the fact that they share similar architecture organization (Kwon and Workman, 2011). We suggest that the specificity lies in the role of the "Extended hinge" domain that is able to discriminate between Asf1a and Asf1b. Therefore, we propose that it is the composition of Asf1a and Asf1b complexes that governs the targeting of each HP1 isoform. Of course this requires further investigations and several studies are needed to address this question.

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