DISSERTATION

STRUCTURAL INSIGHTS INTO CHROMATIN ASSEMBLY FACTOR 1 AND NUCLEOSOME ASSEMBLY MECHANISM

Submitted by

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ABSTRACT

STRUCTURAL INSIGHTS INTO CHROMATIN ASSEMBLY FACTOR 1 AND NUCLEOSOME ASSEMBLY MECHANISM

The eukaryotic genome is highly packed with histones to form chromatin. The basic building unit of chromatin is the nucleosome, which consists of a histone octamer core, wrapped by 147 base pairs of DNA. The nature of the nucleosome structure presents a formidable barrier for DNA-related processes, especially for DNA replication. Therefore, the chromatin will undergo dramatic dynamics during replication, involving disassembly of old nucleosomes and distribution of both new and old histones to form nucleosomes onto both daughter DNA strands. These nucleosome dynamics suggest a challenge for the maintenance of histone density and epigenetic inheritance in the wake of DNA replication. Chromatin Assembly Factor-1 (CAF-1) is a conserved histone chaperone that directly interacts with the replication machinery via the polymerase processivity factor PCNA, and is involved in assembling nucleosomes behind the DNA replication fork. CAF-1 is essential for multicellular eukaryotes, while deletion of CAF-1 in yeast is not lethal, but results in increased sensitivity to DNA damage and aberrant telomeric silencing. Despite the significance of this histone chaperone, the structural organization of this complex remains largely unknown, and thus the mechanism underlying CAF-1-mediated nucleosome assembly is elusive. In this study, we identified the key peptides involved in CAF-1 subunit assembly by performing HDX-MS analysis followed by site-directed mutagenesis studies, which were confirmed by yeast genetic studies. This structural information allows us to further characterize functional domains within CAF-1, and provides unprecedented details for future structural studies using crystallization and/or cryo-EM. This work also shows how

histones H3-H4 are bound by CAF-1, and how this histone binding regulates the nucleosome assembly activity by CAF-1. We also show that DNA is acting as a bridge to bring two histone-bound CAF-1 together, thereby promoting $(H3\text{-}H4)_2$ tetramer formation as well as the tetramer hand-off between CAF-1 and DNA, resulting in the formation of tetrasome ($(H3\text{-}H4)_2$ tetramer wrapped with DNA), the initial step for nucleosome assembly. Overall, this study provides a mechanistic explanation for efficient nucleosome assembly by CAF-1 following DNA replication, and highlights a direct nucleosome assembly mechanism by a histone chaperone for the first time. Moreover, the concerted mechanism of CAF-1-mediated nucleosome assembly suggests that two H3-H4 dimers are brought together right before the $(H3\text{-}H4)_2$ tetramer deposition onto DNA, shedding light on the future directions of epigenetic maintenance regulation during replication.

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CHAPTER 1: INTRODUCTION

1.1 Chromatin

1.1.1 Chromatin is the natural form of the eukaryote genome

In human cells, about 2 meters of DNA are packaged within an interphase nucleus that is about 10–20 μm in diameter (Bloom and Joglekar, 2010). This is a huge challenge in terms of 1 million fold compaction, but more challenge comes from the proper organization of such a massive genome considering the highly negative charged DNA fiber. Nature has evolved a great pathway to achieve this. In eukaryotes, a group of highly basic proteins named histones have evolved to neutralize the acidic phosphate of DNA, and package DNA by spooling it around core histone octamers. This massive protein-DNA complex, along with other structural and regular proteins and RNA, is named chromatin (Figure 1.1). The chromatin is acting not only as a storage form for genomic DNA, but also represents an integrative platform for regulatory accessibility to DNA.

The basic unit of chromatin is the nucleosome, which forms a disc-shape structure. The nucleosomes are connected by variable length of linker DNA to form nucleosomal arrays, which are visualized as a “beads on a string” under the electron microscope. Nucleosomes can interact with each other either directly or indirectly through non-histone structural proteins to form higher order structure, which is the chromatin fiber with around 30 nm diameter. Most recent studies using crystallography and cryo-EM demonstrate the 30 nm fibers of nucleosome arrays are organized into a left-handed twist of tetranucleosome repeat, within which linker DNA of four nucleosomes zigzag back and forth in a two-step nucleosome stacking (Schalch et al., 2005; Song et al., 2014). Nevertheless, real chromatin structure in the cell is elusive, and recent progress on chromatin tomography studies *in vivo* reveals that chromatin forms flexible chains

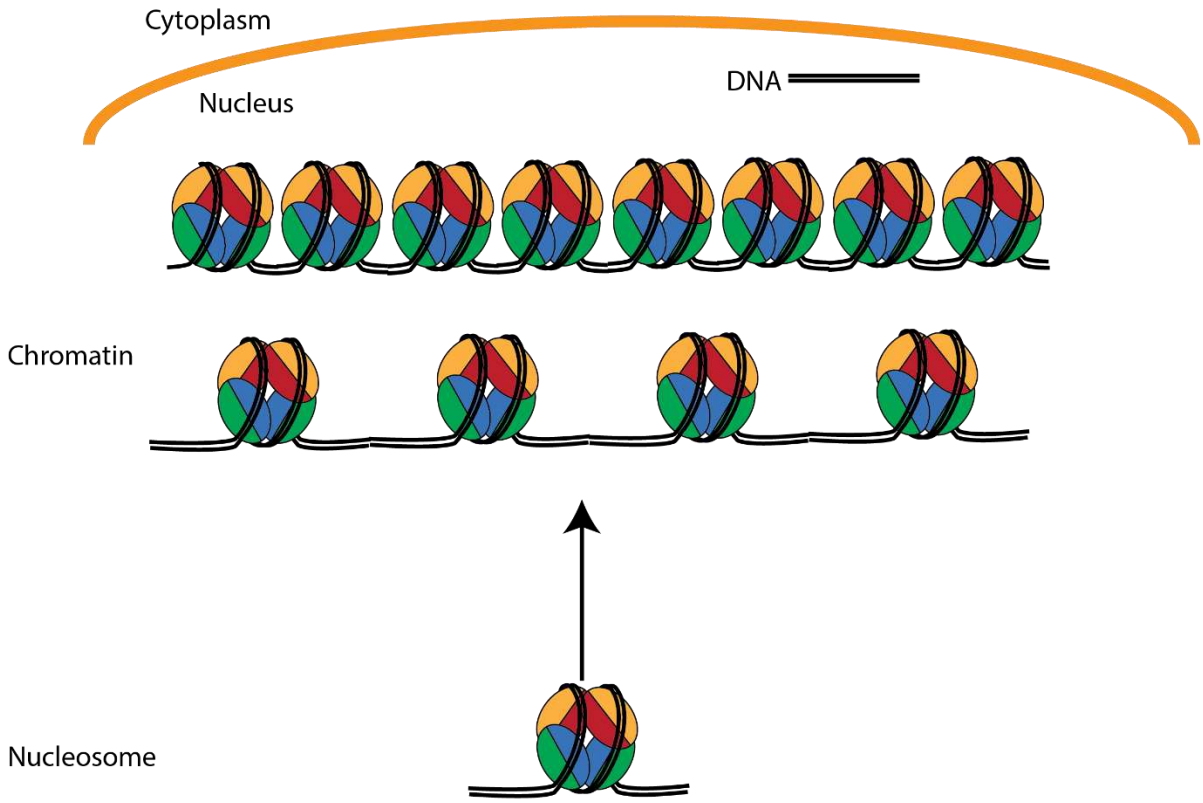

Figure 1.1 Nucleosome is the basic unit of the chromatin. Blue represents histone H3, green represents histone H4, yellow represents H2A and red represents H2B.

with diameters between 5 nm and 24 nm (Ou et al., 2017). This study is compatible with the long-standing concept that chromatin structure is quite diverse across the entire genome, rather than a unified simple 3D structure. The assembly of DNA into chromatin generally excludes access of DNA and RNA polymerases, but also provides extra protection from DNA damage.

1.1.2 Diverse chromatin organization

Chromatin is not randomly packed in the nucleus, rather it adopts distinguished conformations that further regulate the proper function of chromatin. Accumulative evidence showed that the spatial organization of the genome profoundly influences how genes are regulated in normal development, or dysregulated in disease. Chromatin is organized into topologically constrained functional domains. Recent chromosome conformation capture (3C) methods, including updated 4C, 5C or high C provides evidence at high genomic sequence resolution for “topologically associated domains (TADs)” that range between hundreds to thousands of kb DNA (Belmont, 2014; Dixon et al., 2012; Nora et al., 2012). TADs are believed to behave not only as a structural unit, but also as a functional unit of chromatin. These TADs represent chromosomal units within which genomic DNA preferentially forms contacts. These closely associated TADs are usually clustered to form compartments, that are either open and active, or closed and inactive (Denker and de Laat, 2016). The organization of chromatin in diverse compartments provides a way to fine-tune regulation during development.

Chromatin can be condensed further into a rod-shaped structure, which is named chromosome, distinctively visible at metaphase and anaphase during cell division. This compaction is facilitated by a universal protein named cohesin (Hirano, 2000). The further compaction into chromosome at these stages is believed to provide mechanical protection during chromosome segregation.

1.1.3 Heterochromatin and euchromatin

Chromatin is not uniform in terms of its structural organization. In the cells, based on the compaction level at interphase, chromatin has distinctive states: heterochromatin is condensed throughout interphase, while euchromatin is de-condensed (Schultz, 1936). Later on, it was realized that heterochromatin is found mainly in gene-poor regions, but is also prominent in telomeres, pericentric regions and repetitive sequences, while euchromatin is found in active regions. Heterochromatin is also known as heritable “silent” chromatin in yeast, as this region is refractory to gene expression (Gottschling et al., 1990). In yeast, heterochromatin is found at mating type loci, sub-telomeres (a chromosome region adjacent to telomeres) and the rDNA arrays (ribosomal RNA coding region) (Grunstein, 1998). Heterochromatin formation and maintenance integrates diverse information, including genomic integrity, chromatin organization and nucleus localization (Dillon and Festenstein, 2002; Jones and Baylin, 2002; van Steensel and Belmont, 2017). Heterochromatin formation normally requires specific histone modifications and non-histone regulators to distinguish it from other genomic regions. Recent studies suggest a phase separation mechanism for heterochromatin formation (Hnisz et al., 2017; Larson et al., 2017; Strom et al., 2017), which partially explains the sharp transition between heterochromatin and euchromatin, suggesting the compact nature of heterochromatin alone does not account for the steric exclusion of regulatory proteins and RNA polymerase (Elgin and Reuter, 2013).

Overall, the organization of chromatin into heterochromatin and euchromatin demonstrates discrete chromatin landmarks that differentiate the multiple functions. Euchromatin has high genomic accessibility, thus allowing for efficient recruitment of transcription factors and RNA polymerase; while heterochromatin serves as stable and heritable chromatin regions, and provides mechanical stability important for chromosome segregation throughout the cell cycle.

1.1.4 Maintenance of heterochromatin

Reporter genes that are inserted adjacent to telomeres of S. cerevisiae chromosomes are silenced through a process termed telomere position effect (Gottschling et al., 1990).

In budding yeast, heterochromatin is mainly found at mating type loci, the telomeres and the rDNA arrays (ribosomal RNA coding region). Formation of silent chromatin at these three loci serves different purposes and requires different protein components (Rusche et al., 2003). The assembly of chromatin into heterochromatin structures at the silent mating loci is accomplished by the recruitment of the Silent Information Regulator (Sir) proteins to the silencers, the *cis* DNA sequences that dictate the silenced loci. SIR family proteins are involved in histone deacetylation and act as scaffold proteins in the condensation of chromatin in these regions. The spreading of Sir proteins are terminated by boundary elements at the mating type loci (Donze and Kamakaka, 2001; Suka et al., 2002). The DNA sequence dependent silencing in mating type loci seems independent of DNA replication, as studies show that silent heterochromatin still could form on DNA that does not contain a replication origin (Kirchmaier and Rine, 2001; Li et al., 2001). It is also shown that other regulators such as histone chaperones (discussed in the following chapter) is involved in recruiting the SIR family proteins. Histone chaperone mediated heterochromatin maintenance takes place mainly in the subtelomeric regions (Huang et al., 2005; Huang et al., 2007; Kaufman et al., 1997; Monson et al., 1997), which will be discussed in more details in later chapters.

In fission yeast, these heterochromatin regions are highly associated with H3K9 methylation and histone deacetylation. Histone H3K9 is methylated by the methyltransferase Clr4. H3K9me provides a binding site for the heterochromatin protein Swi6, the sequence and functional homolog of heterochromatin protein 1 (HP1), a prominent heterochromatin marker in higher eukaryotic cells. Thus, proper heterochromatin maintenance involves the establishment of this modification pattern after replication, and heterochromatin protein recruitment afterward. Similar

to the budding yeast, Sir2 is also involved in heterochromatin formation and maintenance by creating hypoacetylated histones in these regions (Shankaranarayana et al., 2003). Interestingly, RNA interference plays another important role in regulating heterochromatin maintenance by balancing the replication and transcription activity around the replication origin, where the replication machinery initiates on the genome. The replication machinery opens up the heterochromatin, allowing for transient transcription, whose transcripts are processed into small interfering RNA (siRNAs) that accumulate during S phase. This RNAi-dependent machinery targets to repetitive DNA elements, and further recruits the lysine methyltransferase (Clr4) to modify the newly deposited histones following replication. In animals and plants, it is still unclear if similar RNAi-dependent heterochromatin maintenance mechanisms exist.

However, heterochromatin in animals and plants is not only annotated by H3K9 methylation, but also by DNA methylation. The crosstalk between DNA methylation and histone methylation might facilitate the proper heterochromatin maintenance during replication. DNA methylation is faithfully maintained by a semi-conservative mechanism which is well established. The methylated DNA would become semi-methylated after DNA replication with incorporation of un-methylated DNA. This semi-methylated DNA is recognized by a SET- and RING- associated (SRA) domain containing protein (Arita et al., 2008; Avvakumov et al., 2008), NP95, which directs the DNA methyltransferase 1 (DNMT1) locally to retain the fully methylated DNA (Sharif et al., 2007). The proper maintenance of methylated DNA might serve as a platform to further modify related histone modifications. For example, methyl CpG-binding protein 1 (MBD1) can be accumulated at the DNA methylation rich region, and it can recruit the lysine methyltransferase SETDB1 to propagate H3K9 methylation along the newly deposited histones (Sarraf and Stancheva, 2004). By either mechanism, chromatin assembly, which is involved in maintaining the histone density on the newly synthesized DNA, is the essential and fundamental process for proper heterochromatin maintenance. In my thesis study, I want to understand how

the histones are deposited during DNA replication, an essential step for maintaining genomic integrity and heterochromatin inheritance.

1.1.5 The functions of chromatin

Chromatin plays essential roles to organize genomic DNA compactly within the nucleus, and to protect the DNA structure and sequence from damage. This compaction is also important for genomic segregation and chromosome structure maintenance during mitosis or meiosis. The compaction of chromatin in general is believed to present a native barrier for multiple DNA-associated processes like DNA replication or transcription, but it also provides another layer of regulation for such accessibility. Moreover, chromatin is closely associated with the inner nuclear membrane through the nucleus pore complex (NPC) and nuclear lamina (NL), a fibrous layer. These lamina-associated domains (LADs) of chromatin are largely composed of heterochromatin that is in a condensed status, thus potentially strengths the nuclear envelope in a mechanical way, suggesting a non-genomic function of chromatin (Bustin and Misteli, 2016)

1.1.6 Summary

In a nutshell, chromatin is involved in the packing of genomic DNA in eukaryotes, and conveys the genomic accessibility under the context of combinatorial interactions between histones, non-histones and DNA. The organization of chromatin structure presents a formidable barrier for DNA-related processes, but the restricted access also allows for fine-tuned regulation of DNA replication and transcription.

1.2 Nucleosome

1.2.1 Nucleosome is the structural unit of chromatin

The nucleosome is the repeating unit of chromatin, which consists of a histone octamer wrapped around by 147 bp DNA with 1.7 turns (Luger et al., 1997) (Figure 1.2). The nucleosome structure is remarkably conserved across species (Andrews and Luger, 2011). A histone octamer contains two copies each of histone H2A, H2B, H3 and H4. At the center of the

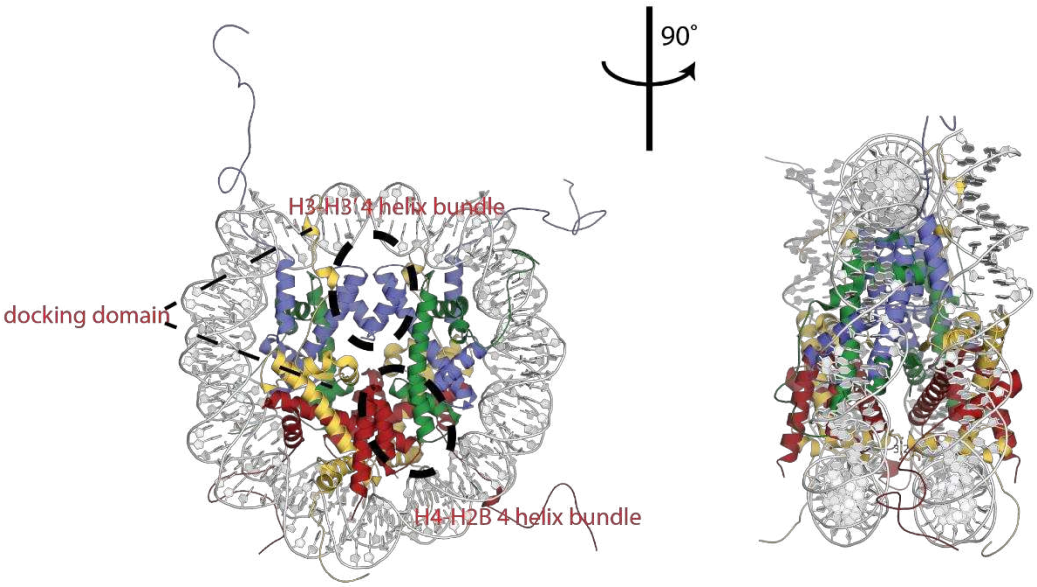

Figure 1.2 Structure of nucleosome particle. PDB: 1aoi. The DNA is shown as pale white. The histone H3 is shown as in blue. H4 is colored in green. H2A is colored in yellow, and H2B is colored in red.

nucleosome, there is a $(H3\text{-}H4)_2$ tetramer associated with ~65 bp DNA (tetrasome). The tip of the tetrasome is at the center of pseudo-symmetric nucleosomal DNA, which is termed the nucleosome dyad. Two H3-H4 dimers form a four-helix bundle structure through their C termini. Two H2A-H2B dimers each associate with around 30 bp DNA, and are docked on either side of the tetrasome, assembling nucleosomes in a disc-like shape. H2B and H4 form another four-helix bundle at their C termini, but with less stability compared to the H3-H3' one. H2A binds to another pair of H3-H4 dimer through its docking domain and its C terminal tail (aa 80-119). The docking domain is important to organize the α N helix of H3 to interact with DNA. Each histone dimer pair of the histone octamer binds to DNA, which spans 2.5 turns of superhelical DNA, generating a 140° bending arch of the DNA, with 3 contacts at the minor groove of DNA facing inward. Overall, the histone octamer has 14 relatively weak contact points with DNA, but combined they provide strong positional stability, and make the nucleosome a very stable complex under physiological conditions. This stability makes the nucleosome resistant to many endogenous nuclease, such as micrococcal nuclease (MNase). Unstructured histone tails protrude through the gyres of the DNA superhelix, and potentially regulate the inter-nucleosome interactions in higher chromatin structure organization. At the entry and exit site of the linker DNA from nucleosome, there is a special linker histone H1. Along with the linker DNA, the linker histone helps stabilize the packing of nucleosomes in higher order chromatin structure.

1.2.2 Histone: intimate partners of DNA

Histones are one of the most conserved class of proteins. Histones are highly basic proteins, with a pI above 10. Histones are also very small (~11-14 kDa), and are prone to interact non-specifically and aggregate with many other macromolecules within the cell. All four histones in the nucleosome share a conserved histone fold domain, which consists of three helixes linked by two flexible coils. Besides the conserved histone fold domains, histones also contain various lengths of unstructured tails which are subject to diverse post-translational modifications

(PTMs), and are important for regulating inter-nucleosome interactions as well as trans-acting factor recruitment. Histones are strongly paired either in H2A-H2B or H3-H4 heterodimers, with two histone fold domains forming a stable “shake-hand” structure with a “head to tail” orientation (Arents et al., 1991). Besides the histones in the nucleosome core particles, there is a linker histone H1, which contains a globular domain rather than a histone fold domain.

1.2.2.1 Histone variants

During evolution, histone variants have emerged to be involved in specific functions. For example, H3.3, a H3 histone variant, is involved in transcription regulation, and highly dynamic H3.3 exchange is mainly found in active genes. CENP-A, another H3 histone variant, is involved in defining localization of the centromere, which is an essential structure for proper chromatid segregation during mitosis. Interestingly, histone H4 and H2B have barely any known variants compared to their partner histone H3 and H2A.

1.2.2.2 Histone modifications

Histones can be post-translationally modified, either before incorporation into chromatin or while located on chromatin. Post-translational modifications (PTMs) of histones regulate many DNA-templated processes, including replication, transcription and repair (Bannister and Kouzarides, 2011). PTMs can directly modulate the chromatin structure or recruit readers that contain PTM-specific binding domains (Bannister and Kouzarides, 2011; Campos and Reinberg, 2009; Tessarz and Kouzarides, 2014). For example, the chromodomains of PRC complexes (Polycomb Repressive Complex) bind to H3K27me2/me3, whereas the chromodomains of HP1 bind to H3K9me2/me3 (Jacobs and Khorasanizadeh, 2002; Min et al., 2003). The specificity between PTMs and their readers defines the root of the hypothesis of the histone code (Strahl and Allis, 2000). For example, hypoacetylation, H4K20me3, and H3K27me3 are marks typical of silenced chromatin, whereas hyperacetylation, H3K4me3, and H3K36me3 are hallmarks of actively transcribed chromatin (Campos and Reinberg, 2009).

Histone modifications do not act in isolation, but they appear to act in concert with each other or even together with underlying heritable DNA methylation. This complexity of combinational regulation between different modifications might benefit the development in higher eukaryotes, which have more diverse histone modifications and histone variants than unicellular eukaryotes (Ruthenburg et al., 2007).

Newly synthesized histones are also specifically modified before being incorporated into chromatin. Multiple H4 acetylation and H3 methylation has been observed on mammalian new histones, while H3K56ac is a hall mark of new histones in yeast (Han et al., 2007; Recht et al., 2006; Sobel et al., 1995). However, it is not clear whether such a distinction between new and old parental histones has a role in chromatin maintenance during DNA replication (more details in the next chapter).

Given the multiple histone variants, as well as the large number of distinctive post-translational modifications, nucleosomes are quite diverse throughout the chromatin. This variability affects the landscape of chromatin and interactions of nucleosome with non-histone proteins, providing the basis for gene regulation and cell fate decision.

1.2.3 DNA preference in nucleosome formation

Each nucleosome contains ~146 bp DNA, which is bent and wrapped around the histone octamer. This uniform bending occurs at every helical repeat, which is around ~10 bp DNA. Thus, different from other DNA-binding proteins that achieve specificity by direct strong interactions between several base pairs of DNA and the amino acids, the binding affinity of histone to DNA is largely dependent on the ability of bending for the DNA (Drew and Travers, 1985). DNA sequences differ in their ability to bend (Widom, 2001), and it was modeled that an ideal DNA sequence for wrapping a nucleosome contains 10 bp periodic AA/TT/TA dinucleotides that oscillate in phase and 10 bp periodic GC dinucleotides that are out of phase (Segal et al., 2006). Therefore, the ability of histone octamer to wrap DNA into a nucleosome is

indirectly dependent on the specific DNA sequence. Through *in vitro* studies using SELEX John Widom and his colleagues isolated a high affinity histone binding DNA sequence, which is named “601” sequence, that shows over 1000 times stronger affinity than some natural DNA sequences (Lowary and Widom, 1998). The dramatic energy difference for specific DNA sequence to bind to histones provides a unique and efficient way to reconstitute well-positioned nucleosomes *in vitro* by using “601” sequence.

In vivo, the DNA preference for nucleosomes would impact the nucleosome positioning in certain loci. More importantly, nucleosome positioning is refractory to DNA-related processes. For example, the replication origin in eukaryotes is defined as short ARS (autonomously replicating sequence) consensus sequence (ACS), which contains 11 bp that are AT-rich. This sequence is not ideal for stable nucleosome assembly, and thus most of the ACS is a nucleosome free region (NFR) *in vivo* (Eaton et al., 2010). Similar to the replication origin, poly A (or poly T) tracts of DNA sequence are important for nucleosome depletion in the promoter and terminator regions of many genes (Kaplan et al., 2009). Although DNA sequence plays important roles for nucleosome positioning *in vivo*, the actual nucleosome position footprint is influenced by many regulatory factors such as chromatin remodelers, transcription factors and polymerase (Struhl and Segal, 2013).

1.2.4 Nucleosome dynamics

As discussed above, nucleosomes present a general barrier for DNA-related processes, such as DNA replication or transcription. Therefore, during the passage of DNA polymerase or RNA polymerase, the nucleosome has to be transiently disrupted. Many factors are involved in the regulation of nucleosome dynamics during these processes, including DNA helicases, chromatin remodelers as well as histone chaperones. The nucleosome dynamics are generally grouped into four categories: DNA breathing, histone turnover, nucleosome remodeling, and nucleosome assembly.

1.2.4.1 DNA breathing

The nucleosome crystal structures only represent one possible state of the nucleosome (Andrews and Luger, 2011). The spontaneous partial unwrapping and re-wrapping of the DNA from nucleosomes as a consequence of thermal dynamics is a process known as DNA breathing (transient dissociation between DNA and histone) (Blossey and Schiessel, 2011; Li et al., 2005). DNA breathing has been observed *in vitro* by using single molecule FRET (smFRET) upon changing the salt conditions of the nucleosome environment or diluting nucleosomes concentrations to low nanomolar range (Gansen et al., 2009; Koopmans et al., 2009). The transient unwrapping and rewrapping of nucleosomal DNA ends has been identified on a timescale of 1–10 ms by using smFRET combined with bulk stochastic data analysis (Wei et al., 2015). DNA breathing provides a transient chance for trans-acting regulators to bind to sites otherwise hidden by the histone-DNA interface (Anderson and Widom, 2000; Tims et al., 2011). This breathing mechanism is also thought to create DNA loops, where the nucleosome re-associates with DNA further along the sequence. This has the potential to allow nucleosome translocation without dissociation from the DNA during processes such as RNA polymerase traverse (Anderson et al., 2002).

This breathing may be promoted by histone chaperones *in vivo*, such as FACT (facilitates chromatin transcription) and nucleosome assembly protein (Nap1), by histone acetylation and/or by sequence-specific DNA-binding proteins, whose cognate sites reside just within the entry and exit sites (Lai and Pugh, 2017).

1.2.4.2 Histone turnover

Histones are removed and then replaced on DNA as a consequence of transcription or DNA replication. Histone turnover has been shown to change during differentiation, suggesting that histone turnover correlates with DNA accessibility (Deaton et al., 2016). Histone turnover has been measured by attaching an epitope (such as a SNAP-tag) to histones and tracking the

labeled histone abundance on chromatin in a time series after pulsing (Clement et al., 2016). Histone turnover rate varies across the genome. For example, histone variant H3.3 has high turnover rate in enhancers, particularly in super enhancers, and histone variant H2A.Z has high turnover rate at the +1 nucleosome, the very first nucleosome following the TSS (Transcription Start Site); while the histones in silent chromatin generally have lower turnover rates.

Histone variants are incorporated to specific loci via histone exchange with canonical histones for specific regulation. For example, H2A.Z might destabilize nucleosomes and thus allow RNA polymerase II to penetrate them more readily, thereby allow for efficient histone turnover (Suto et al., 2000; Zhang et al., 2005a).

Nucleosome dynamics have been studied to demonstrate the stability of nucleosomes, or in other words, the resistance to external disturbance. Single molecular fluorescence has been used to study the DNA dynamics on the nucleosome (Bohm et al., 2011), while SYPRO orange is used to test the thermal stability of nucleosomes (Taguchi et al., 2014).

1.2.4.3 Nucleosome remodeling

The histone octamer could slide along DNA, a process promoted by ATP-dependent chromatin remodelers (Clapier and Cairns, 2009; Narlikar et al., 2013). *In vivo*, the sliding of nucleosomes results in reshaping of the chromatin landscape on the genome. Nucleosome remodeling plays important roles in regulating the accessibility of the genome otherwise tightly compacted during DNA replication, transcription as well as DNA repair (Narlikar et al., 2013). For example, Chd1 is involved in placing regularly-spaced nucleosome arrays and regulates transcription elongation in yeast (Hennig et al., 2012; Pointner et al., 2012). Single molecule studies of the nucleosome remodeling dynamics indicate that the nucleosome remodeling generally happens in a 3-step manner: first, part of nucleosomal DNA is dislodged from histone octamer; second, the linker DNA is sliding along the histone octamer with the help from ATPase activity of remodeling complex, generates new contacts between DNA and octamer; finally the histone octamer is

repositioned along DNA (with new contacts for each histone-DNA contact points within the nucleosome) (Deindl et al., 2013; Narlikar et al., 2013). Recent structural studies suggest that histone octamer conformational changes can distort the DNA-histone contacts, so chromatin remodelers can regulate the DNA sliding along the histone (Sinha et al., 2017).

Nucleosome dynamics also includes their de novo assembly, which will be discussed in more detail in the following section.

1.2.5 Nucleosome assembly

Nucleosomes are assembled without energy input from ATP. During each round of DNA replication, millions of nucleosomes will be assembled to maintain chromatin structure. However, the molecular mechanism of this process is unclear.

1.2.5.1 Nucleosome reconstitution

Nucleosome core particles can be reconstituted *in vitro* with isolated histones and DNA by salt dialysis, as established over 40 years ago (Tatchell and Van Holde, 1977). The formation of nucleosomes through salt reconstitution is believed to occur in a two-step process, during which H3-H4 associates with DNA at higher salt conditions, and H2A-H2B is incorporated when the salt concentration is decreasing (Burton et al., 1978). Nucleosomes could also be reconstituted simply by splitting the gradient salt dialysis into several steps of salt dilution (Dyer et al., 2004; Muthurajan et al., 2016). This dramatically increases the nucleosome reconstitution and optimization efficiency. The efficient nucleosome reconstitution paves the roads for studying the biophysical properties of the variant homogeneous nucleosome samples.

1.2.5.2 *In vivo* nucleosome assembly

However, no salt gradients are established when nucleosomes are assembled *in vivo*, which happens at the physiological salt condition. As such, nucleosomes are assembled with the assistance of a group of proteins that include histone chaperones and chromatin remodelers.

Nevertheless, nucleosome assembly necessarily also follows a two-step pathway (Figure 1.3), during which tetramer is deposited onto DNA first (I call this step tetrasome assembly in the remainder of this thesis), followed by incorporation of two H2A-H2B dimers in a later stage. There are basically two hypotheses regarding tetrasome assembly, which is the initial but also essential step for the nucleosome assembly. In the first scenario, as H3-H4 is in a tetrameric form within the nucleosome (Luger et al., 1997), histone H3-H4 could form a tetramer before association with DNA, suggesting that histone chaperones might assemble tetramers before handing them off to DNA. In the second scenario, newly synthesized histones H3-H4 are mainly in a dimer form in the nucleus before being deposited onto chromatin, suggesting that histone chaperones assemble tetrasomes by handing off one H3-H4 dimer after another. H2A-H2B are then assembled onto tetrasomes with the help of specific H2A-H2B histone chaperones.

The two H2A-H2B dimers have limited contact with each other within nucleosomes, thus there might not be too much crosstalk between them during their incorporation.

1.2.5.2.1 Replication-independent and –dependent nucleosome assembly

In vivo, there are mainly two types of nucleosome assembly based on their coupling to DNA synthesis: replication-dependent nucleosome assembly and replication-independent nucleosome assembly.

Replication-independent nucleosome assembly is mostly associated with transcription. This includes partial nucleosome disassembly and reassembly following the passage of RNA polymerase. Some histone H3 variants, such as H3.3 and CENP-A, are assembled into nucleosomes in a replication-independent manner, while the majority of ‘major-type’ histones are assembled in the replication-dependent pathway. Most nucleosome assembly in the cell cycle happens mainly in a small time window during DNA replication. Nucleosome assembly is essential to maintain the chromatin structure after DNA replication, however the mechanism by which this occurs is unclear. In my thesis, I am studying the histone chaperone CAF-1, the

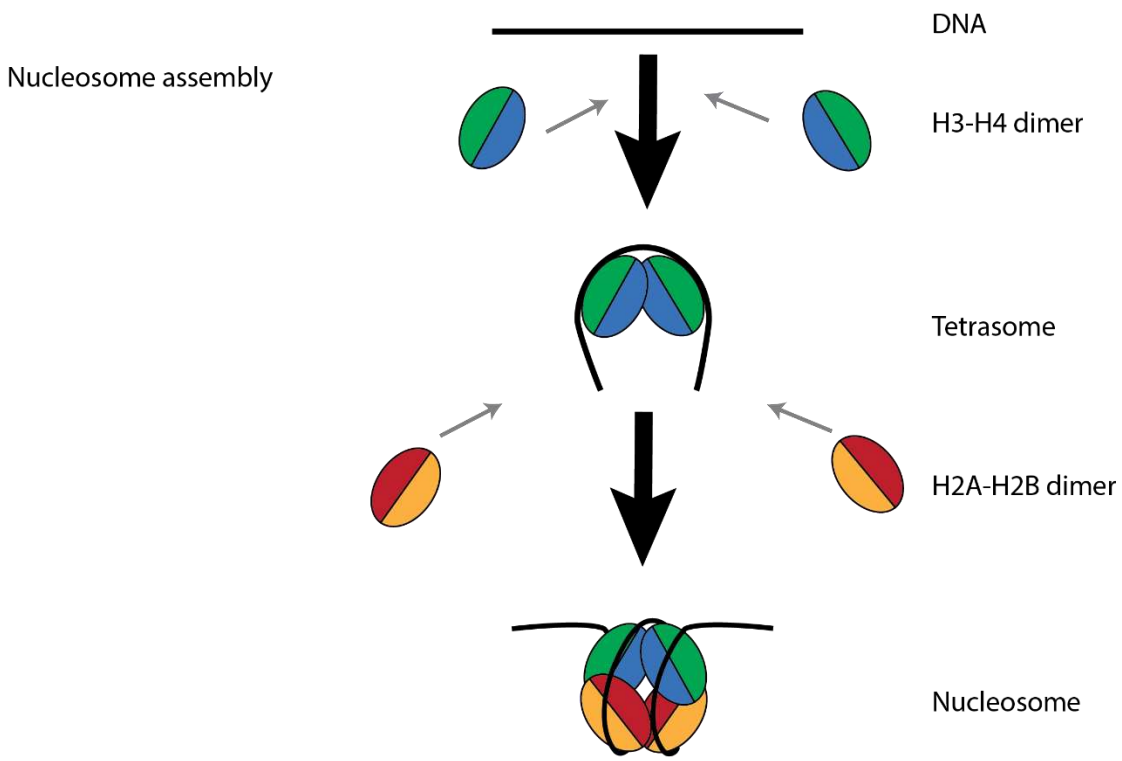

Figure 1.3 A general model for nucleosome assembly. Black line represents the DNA. Histone H3 is colored in blue, and H4 is colored in green. Histone H2A is colored in yellow, and H2B is colored in red.

major player of nucleosome assembly *in vivo*, to study the mechanism of nucleosome assembly. Since replication-coupled nucleosome assembly is the majority, I specifically look at the nucleosome assembly which happens during replication. Accumulating evidence suggest that in DNA replication, nucleosome assembly is highly coupled to the replication machinery, suggesting the timely chromatin restoration on the newly synthesized DNA is important for the replication progress (Groth et al., 2007b; Kaufman et al., 1995) (Groth et al., 2007a; Jasencakova et al., 2010). In the next chapter I will introduce the replication machinery, and how DNA replication coordinates with chromatin structure.

1.3 DNA Replication

The genomic DNA needs to be replicated for the maintenance and proliferation of life. The DNA replication mechanism is highly conserved basically in all domains of life, which follows a semiconservative manner based on the unique pairing of bases (Watson and Crick, 1953). The DNA replication in eukaryotes is controlled in the context of the cell cycle, which happens within S phase (synthesis phase). The DNA replication in eukaryotes mainly involves four steps: replication initiation, replication fork assembly, replication elongation and termination. All of these steps occur in the context of chromatin.

1.3.1 Brief overview of DNA replication

Replication initiation involves in the recognition of the replication origin as well as the assembly of the pre-replicative complex (pre-RC). This pre-RC complex contains a six subunit ATPase, which is called the Origin Recognition Complex (ORC). In budding yeast, origins of DNA replication were defined as short ARS (autonomously replicating sequence) consensus sequence (ACS). But the ARS sequence alone does not determine the replication origins, suggesting that chromatin plays roles in the recruitment of the preRC, either through histone modifications or through local chromatin organization.

After preRC assembly on the replication origin, the licensing cofactor Cdt1 and ATPase Cdc6 are required to recruit two DNA helicase complexes, MCM2-7, which is composed of 6 subunits. Either of helicase complex form a ring on a single stranded DNA. The MCM complex provides the platform to recruit DNA polymerase and other regulators, including Cdc45 and the GINS complex. Subsequent MCM helicase activation depends on the association of Cdc45 and GINS, which together is called CMG complex. The CMG complex provides the force to unwind double stranded DNA to allow single strand DNA to serve as a template for DNA polymerase, concurrently, breaking up double stranded DNA and generating over-twisted DNA ahead, which is potentially the driving force of disassembling nucleosomes before these supercoils get released by topoisomerases. This initial replicative complex synthesize certain length of DNA before an important regulator is loaded by Replication factor C (RFC) to boost the processivity of DNA polymerase (Waga and Stillman, 1998). This processivity factor is identified as Proliferating Cell Nuclear Antigen (PCNA), a homo-trimer complex forming a sliding clamp on the DNA. The loading of PCNA onto the replication fork also replaces pol α with either Pol ∆ or Ɛ, which carry out the majority of DNA synthesis. The incorporation of PCNA and processive DNA polymerase makes a mature replication fork for replication elongation. Each replication fork contains two newly synthesized strands and a parental DNA under unwinding. The strand that is synthesized in the same direction as the replication fork moving is called the leading strand, which is conceivably replicated continuously, whereas the strand synthesized in the opposite direction is called lagging strand, which is replicated discontinuously. Interestingly, lagging strand and leading strand priming are both coordinated through a protein interaction hub Ctf4 (AND-1 in human), to guarantee harmonious pace on both strands (Simon et al., 2014; Villa et al., 2016). However, the discontinuous replication generates various short lengths of DNA, which are named Okazaki fragments (Okazaki et al., 1968).

The total length of human genome is over 3 billion base pairs of DNA, while DNA replication rate in eukaryotes is estimated to be ~60 bp per second. Therefore, tens of thousands of replication origins are firing in a regulated manner to make sure replication could be accomplished in a confined time. Each replication origin fires bi-directionally, creating two replication forks, each with one leading strand and one lagging strand. Replication termination for the leading strand happens when two replication forks converge from two adjacent origins (Dewar et al., 2015). However, lagging strand synthesis is terminated before encountering the adjacent replication machinery. Due to the opposite synthesis direction on the lagging strand, the Pol ∆ which associates with PCNA for the elongation of the lagging strand synthesis, will encounter the primer RNA at the end of elongation. The priming RNA is displaced by an enzyme named FEN 1, and replenished with DNA by Pol ∆. This process is also called nick transition, as the nick is shifting along the lagging strand during the displacement. This nick transition is terminated by the action of DNA ligase, which also interacts with PCNA for efficient recruitment. To what extent this nick transition would continue is potentially dependent on the nucleosome occupancy ahead of the pol ∆ on the lagging strand. Thus, the length profiling of Okazaki fragments has been shown to reflect the nucleosome repeat on the lagging strand (Smith and Whitehouse, 2012), which provides a tool to test the replication-coupled chromatin assembly activity *in vivo*.

1.3.2 Chromatin regulates DNA replication

Since the genomic DNA is packed into a chromatin structure, the chromatin itself has evolved a profound impact over the DNA replication through every stage.

1.3.2.1 Initiation

Unlike in bacteria, whose replication origin is simply defined by DNA sequence, the replication origin in eukaryotes is rather defined in a chromatin context. Nucleosome occupancy at the ACS is refractory to pre-RC assembly, therefore at a functional DNA replication origin, a specific DNA

sequence has to be present to define a nucleosome free region (NFR) for efficient pre-RC assembly (Eaton et al., 2010). The length of the NFR is longer than what is seen in general promoters, a DNA sequence that recruits transcription factors and RNA polymerase for transcription.

Besides that, histone modifications around the replication origin are shown to regulate its activity. For example, in Drosophila, the active replication origin is usually located around chromatin with hyperacetylated histones (Aggarwal and Calvi, 2004).

1.3.2.2 Elongation

The elongation rate in eukaryotes is significantly slower than in prokaryotes, which do not have a chromatin-based genome. Chromatin is not totally disassembled for replication, but only locally disassembles nucleosomes right before the replication fork. Actually, the helicase is involved in the disassembly of nucleosomes during the replication fork progress. Previous studies using optical tweezers to unzip the DNA duplex *in vitro* indicate that the unzipping force from helicase might be sufficient to provoke histone eviction during replication (Shundrovsky et al., 2006). Nevertheless, histone chaperones and chromatin remodelers (discussed later) are involved in coordinating the progress of the replication fork.

1.3.2.3 Termination

As mentioned above, each DNA replication fork contains a leading and lagging strand. In terms of leading strand, DNA replication terminates when replisomes converge from adjacent replication origins. On the other hand, lagging strand synthesis is largely affected by the chromatin which is newly synthesized (Smith and Whitehouse, 2012).

1.3.3 Chromatin re-organization during DNA replication

As mentioned above, chromatin conveys complicated genomic regulation by shaping distinctive structures or landscape along the genome, by introducing histone variants and distinctive PTMs

of histones. The maintenance of chromatin structure is thus important for genome integrity as well as stable cell status during cell division. However, such a process becomes a challenge when DNA undergoes replication.

When the replication fork moves forward, the unwinding of the parental double stranded DNA would cause positive supercoils of DNA ahead of replication fork. This DNA distortion might be the driving force to disassemble the parental nucleosomes (Figure 1.4). *In vitro* studies indicate that the positive torsional stress ahead of the replication could induce a structural change in nucleosome to aid its disassembly (Bancaud et al., 2006). H2A-H2B is initially depleted from nucleosomes first, followed by the dissociation of $(H3\text{-}H4)_2$ tetramers from DNA. Moreover, histone modifications might also facilitate the chromatin decompaction and nucleosome disassembly. For example, histone H1 is phosphorylated by Cdk2, which travels along the replication machinery, so that H1 becomes more dynamic and promotes the decompaction of chromatin (Alabert and Groth, 2012).

Both parental histones are recycled and re-incorporated onto the new daughter strands (Figure 1.4). Meanwhile, newly synthesized histones are transiently expressed and deposited onto the new strands as well to prevent histone density loss after DNA replication. As parental histones have distinctive PTMs from newly synthesized histones, their proper segregation might be regulated to ensure proper epigenetic maintenance during replication. Current studies suggest that parental and new H2A-H2B is incorporated onto chromatin in a stochastic manner (Gruss et al., 1993), and that most post translational modifications regulating genes are found at the N terminal tails of histone H3 and H4 (Lawrence et al., 2016), highlighting proper histone H3-H4 segregation is important for the epigenetic maintenance.

1.3.3.1 Conservative vs semi-conservative segregation of histone H3-H4

A conservative histone segregation mechanism suggests that the parental $(H3\text{-}H4)_2$ tetramers are not split during their transition to daughter strand DNA. Multiple studies observed that

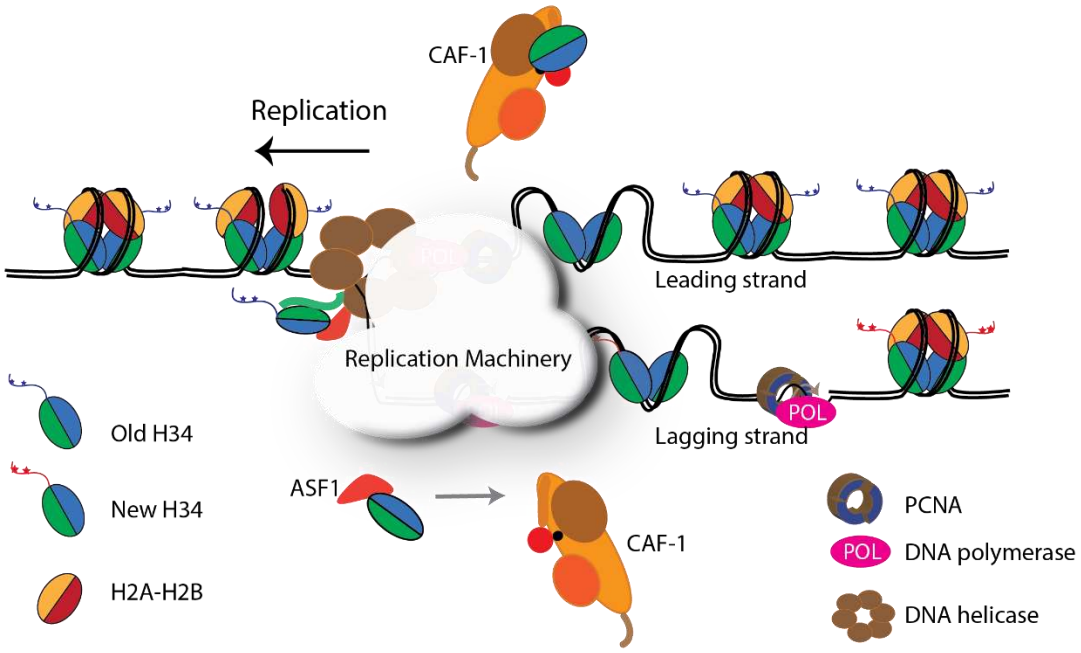

Figure 1.4 Chromatin dynamics under replication. The DNA replication is driven by the replication machinery, which include the helicase, DNA polymerase and ligase. The replication machinery facilitates the disassembly of nucleosomes ahead of the replication fork. Old and new histones are captured by histone chaperones. PCNA is an important platform to recruit histone chaperones for proper nucleosome assembly after DNA replication.

parental histone H3-H4 are segregated onto both new DNA strands in such a manner that the parental histone H3-H4 incorporation is exclusive with new histones H3-H4 in a single nucleosome particle (Prior et al., 1980; Xu et al., 2010; Yamasu and Senshu, 1990). On the other hand, *in vivo* immunoprecipitation suggests that H3-H4 are bound to histone chaperones in a dimer form before they are deposited onto DNA, suggestive of a semi-conservative distribution of histones during cell division (Tagami et al., 2004).

1.3.3.2 Symmetric dispersive vs asymmetric distribution

Symmetric dispersive distribution of histone means that parental histone H3-H4 are evenly distributed to both strands of new DNA, which indicates that H3-H4 are randomly deposited onto both new DNA. In contrast, asymmetric histone distribution suggests that parental H3-H4 are biased in their incorporation onto either leading or lagging strand.

The tight coupling between nucleosome assembly and DNA replication requires tight coordination between histone demand and supply in the nucleus. In mammals, histones in excess of cellular requirements are degraded in a Rad53- and proteasome- dependent manner (Gunjan and Verreault, 2003; Singh et al., 2009). However, low supply of histones of H3-H4 would delay S phase progress, and activate DNA damage check point (Groth et al., 2007a).

1.3.4 Coordinated chromatin re-organization during replication requires cooperative function of many factors

As DNA replication in eukaryotes faces so many challenges and crossroads in the context of chromatin, many factors have evolved to regulate and coordinate the chromatin re-organization process and epigenetic maintenance. These include DNA helicases, polymerase co-factor PCNA, ATP-dependent chromatin remodelers and histone chaperones.

1.3.4.1 MCM helicase complex

MCM helicase is found in eukaryotes and archaea, but not in prokaryotes. As both eukaryotes and archaea contain nucleosomes to compact genome (Forsburg, 2004), the coexistence of MCM helicases and nucleosomes indicates the importance of MCM helicase for nucleosome dynamics. MCM helicase is localized at the front edge of replication fork and its main function is to unwind non-replicated DNA. Mammalian MCM helicase is a six-subunit complex containing MCM2-MCM7. Subunit MCM2 is also involved in capturing histone H3-H4 when they are evicted from parental nucleosomes (Clement and Almouzni, 2015; Huang et al., 2015a).

1.3.4.2 PCNA

PCNA belongs to the family of DNA sliding clamps, which are structurally and functionally conserved among different species (Moldovan et al., 2007). Although the sequence of PCNA is not well conserved across different species, their structures are almost superimposable (Krishna et al., 1994). PCNA forms a ring-shape complex on DNA, which encircles the DNA and could slide freely along DNA. PCNA is loaded onto DNA by the RFC complex in the presence of ATP (Majka and Burgers, 2004). PCNA ensures high processivity of DNA polymerase by tethering it onto DNA when sliding during DNA synthesis. Moreover, PCNA acts as a “platform” that recruits multiple proteins in coordination with DNA synthesis, such as DNA methyltransferase DNMT1, histone deacetylase HDAC1, chromatin remodeling factor WSTF, and histone chaperone CAF-1 (more details in the following sections) (Moldovan et al., 2007).

PCNA binding proteins contain a conserved motif known as PIP (PCNA interaction protein) box, however, some of PCNA binding proteins might have additional interacting regions (Maga and Hubscher, 2003). Since PIP containing proteins binds to PCNA through the same regions, most of these interactions are mutually exclusive or competitive (Maga and Hubscher, 2003; Moldovan et al., 2007). However, since PCNA is a homotrimeric complex, it contains three PIP-docking sites, amenable for multiple protein recruitment at the same time. Nevertheless, some

studies suggest that simultaneous binding of multiple partners to PCNA is not necessary as PCNA might contain only one functional binding site, which could sequentially coordinate with each binding partner to fulfill their functions (Dovrat et al., 2014). In summary, PCNA functions as a platform that links chromatin remodelers and histone chaperones for proper chromatin establishment following DNA replication.

1.3.4.3 Chromatin remodeling complexes

Chromatin remodeling factors are a group of protein complex that utilize ATP hydrolysis to change nucleosome conformation, slide nucleosome along DNA, or change the nucleosome composition to regulate DNA access across the genome (Clapier and Cairns, 2009). All chromatin remodelers share common features such as affinity toward nucleosomes, a similar DNA-dependent ATPase domain (composed of DExx and HELICc), and domains that specifically recognize histone modifications. But they also might contain specific domains or subunits that could interact with other trans-acting factors for efficient recruitment. Based on the unique domains within the ATPase domain, chromatin remodeler are categorized into four major families: SWI/SNF, ISWI, CHD and INO80. During DNA replication, SMARCAD1 (yeast ortholog is Fun30) is a SWI/SNF like remodeling complex that directly interacts with PCNA. It can recruit HDAC1 and KAP1 to the heterochromatin sites, and has been shown to regulate proper chromosome segregation and heterochromatin maintenance (Rowbotham et al., 2011). Another remodeling factor, ACF1 (ATP-utilizing chromatin assembly and remodeling factor 1) is required for efficient replication in the heterochromatin region (Collins et al., 2002), potentially by remodeling condensed nucleosomes to allow for passage of replisomes.

1.3.4.4 Histone chaperone

Histone chaperones are another family of proteins which are involved in histone shuttling and finally deposition of histones onto DNA (De Koning et al., 2007). During DNA replication,

multiple histone chaperones are involved in maintaining chromatin structure after each duplication. I would like to discuss histone chaperone in more detail in the next chapter.

1.4 Histone chaperones

As mentioned above, nucleosome reconstitution can be obtained through slow salt dialysis. However, the direct mixings of histones and DNA at physiological salt conditions results in aggregation. Over 40 years ago, Laskey *et al* used a cell-free nucleosome assembly system and isolated a protein named nucleoplasmin from Xenopus egg extract that is responsible for nucleosome assembly independent of ATP hydrolysis (Laskey et al., 1978). They called it histone chaperone, as it prevents the nonspecific interactions between DNA and histones, and leads to nucleosome assembly. From then on, multiple histone chaperones have been isolated, and the functions of histones chaperones are well accepted. Initially, a histone chaperone was defined as a protein factor that has histone binding activity and nucleosome assembly activities independent of ATP (Eitoku et al., 2008). Nevertheless, not all histone chaperones are involved in nucleosome assembly in the cell, but rather function as chaperones to sequester histones in a non-nucleosomal conformation. Thus, in my thesis, I would like to define histone chaperones simply based on their ability to bind histones in a way that prevents nonspecific interactions between histone and DNA.

Interestingly, despite the highly conserved histone fold structure in all four classical histones, no single binding motif could be applied to all histone chaperones. Both structurally ordered folded domains and unstructured flexible regions are involved in histone binding, and they might be involved in the binding to different regions on histones. Moreover, histone variants demands diverse histone chaperones for their proper incorporation onto chromatin. In this chapter, I will focus on the common features found in histone chaperones, and I will group histone chaperones based on their function in nucleosome assembly and histone buffering. In this

chapter, I will mainly discuss the histone chaperones that are involved in the nucleosome dynamics during DNA replication.

1.4.1 Asf1

Anti-silencing function 1 (Asf1) was originally identified as a protein whose overexpression disrupts the silencing of chromatin in *Saccharomyces cerevisiae* (Le et al., 1997). Subsequently, it was isolated in a complex with histones H3/H4 that could facilitate the replication-coupled assembly of nucleosomes by CAF-1 (another histone chaperone) *in vitro* (Tyler et al., 1999), and recognized as a histone chaperone that regulates the flow of H3-H4 histones during S phase (Groth et al., 2005; Mousson et al., 2007). In most plants and animals except for chicken, Asf1 has two isoforms, Asf1a and Asf1b, which share around 70% sequence similarity, mainly in the N terminal region (Mousson et al., 2007). Asf1a is the major isoform and widely expressed in somatic cells, while Asf1b expression is highly restricted to specific tissues (if not specifically designated, Asf1 is regarded as Asf1a afterward). Abolishing the ASF1b gene in mice does not cause severe phenotypes, suggesting that Asf1a is sufficient for normal function in terms of Asf1(Mousson et al., 2007). Interestingly, recent studies also suggested that Asf1b is a necessary isoform for proper proliferation, and the overexpression of Asf1b is correlated with breast cancer (Corpet et al., 2011). RNAi-mediated knockdown of Asf1 (both isoform a and b) impairs S phase progression in human and Drosophila cell lines, while knock out of the ASF1 gene in chicken DT40 cells is fatal (Groth et al., 2005; Sanematsu et al., 2006; Schulz and Tyler, 2006). Asf1 contains a conserved N terminal domain from yeast to humans, which forms an immunoglobin-like domain (Daganzo et al., 2003). Yeast and *C. elegans* Asf1 contain a long C-terminal tail, which is extremely rich in aspartates and glutamates, an acidic stretch commonly found in many histone chaperones (Lewis et al., 2010; Mousson et al., 2007), while the mammalian Asf1 contains a shorter C-terminal tail without the acidic stretch. However, the mammalian Asf1 C tail is subject to phosphorylation by Tousled-like kinases, a process which

regulates histone flow during DNA replication (Klimovskaia et al., 2014; Pilyugin et al., 2009; Sillje and Nigg, 2001). Thus, the acidic property of the C terminal tail might be a common strategy during evolution for the regulation of Asf-1 function. However, deletion of the acidic tail in yeast does not cause a severe phenotype (Daganzo et al., 2003; Umehara et al., 2002), arguing against a conserved function of the acidic tails. It would be interesting for future studies to understand what specifically the acidic region contributes to the proper function of Asf1, as well as to the relationship with its partners (Klimovskaia et al., 2014).

The conserved N terminal Ig-like domain is formed with tandem antiparallel β-sheets assembled in a sandwich shape (Daganzo et al., 2003). Crystal structures of Asf1 in complex with H3-H4 showed that one side of this “sandwich”-shape binds to the C terminal of H3, in a manner that blocks four helix bundle formation between H3-H3’, while the other side of the “sandwich” is a small epitope recognized by the B domain found in other proteins which crosstalk to Asf1 (English et al., 2006; Malay et al., 2008; Natsume et al., 2007; Tang et al., 2006). In yeast, the acidic C terminal tail of Asf1 might also engage in histone binding, as shown in widespread crosslink with H3 within the complex, and by the finding that loss of the entire C terminal tail dramatically decreases Asf1 affinity for histones (Dennehey et al., 2013).

Asf1 functions at the level where histone H3 and H4 are properly folded and paired after synthesis, and is involved in histone import into the nucleus before incorporation into chromatin. Newly synthesized histones are specifically acetylated on histone H4 tails, and on H3K56 in yeast (Sobel et al., 1995), and these modifications are important for efficient shuttling from cytoplasm into the nucleus (Ejlassi-Lassallette et al., 2011). In yeast, Asf1 facilitates the H3K56 acetylation in the cytoplasm in assistance with Rtt109, an acetyltransferase functioning on soluble histones, to facilitate shuttling of H3-H4 from cytoplasm into nucleus during S phase, and to promote its association with other histone chaperones (Li et al., 2008). Meanwhile, Asf1 isolated from the cytosol is in a complex with Importin 4 (nuclear transport receptor), Hat1

(histone acetyltransferase) and other histone chaperones like NASP, RbAp46, RbAp48 (Jasencakova et al., 2010), suggesting it functions in transporting H3-H4 directly in cooperation with other histone chaperones. Moreover, Asf1 binds to the dimeric form of H3-H4, and does not assemble tetrasome per se (Liu et al., 2012). Thus, Asf1 is involved in handling histones over to downstream histone chaperones such as CAF-1, HIRA, DaXX for deposition onto DNA.

1.4.2 MCM2

MCM2 is one subunit of the hexameric MCM helicase complex. Unlike other MCM subunits it contains a long and flexible highly conserved N terminal tail, which is rich in glutamate and aspartate (Foltman et al., 2013). Recent biophysical and structural studies highlight this subunit as an important regulator for parental histone recycling during replication (Clement and Almouzni, 2015). Mutation of this conserved tail in yeast does not affect the DNA synthesis per se, but leads to loss of subtelomeric silencing (Foltman et al., 2013), a similar phenomenon as observed with replication-coupled nucleosome assembly defects (Kaufman et al., 1997). This tail binds to the lateral surface of the H3-H4 dimer, covering the DNA binding interface on H3-H4 as well as directly competing with the docking domain from H2A-H2B dimer (Huang et al., 2015a). This binding mode allows for histones H3-H4 evicted from nucleosome to be captured in proximity, which facilitates DNA unwinding. Since parental histones are recycled onto daughter DNA strand, the next question would be whether MCM2 could form a ternary complex with chaperone FACT (more in the following part) and histones through this conserved tail. Superposition of the two structures (PDB: 4z2m, 5bnv) indicates that FACT and MCM2 bind to distinctive sites on H3-H4 without steric collision based on the truncated proteins, suggesting a co-chaperoning mechanism (Huang et al., 2015a; Tsunaka et al., 2016). As FACT binds to H3-H4 in its tetramer form similar to its structure within the nucleosome (Tsunaka et al., 2016), this co-chaperoning mechanism might suggest conservative segregation of histones H3-H4 after replication. However, on the other hand, as Asf1 could break up the $(H3\text{-}H4)_2$ tetramer, and bind

to MCM2-H3-H4 to form a sandwich-like ternary complex (Richet et al., 2015), this suggests a semi-conservative segregation of histone H3-H4 or at least a transient disruption of the parental $(H3\text{-}H4)_2$ tetramer. Interestingly, blocking DNA synthesis while allowing parental DNA unwinding causes accumulation of the MCM2-H3-H4-Asf1 complex (it is not clear about the FACT-H3-H4-MCM2 complex level change) (Groth et al., 2007a), arguing that at least Asf1 is involved in the parental histone recycle. It would be interesting to investigate whether both mechanism are existing in parallel under the context of histone recycle.

1.4.3 FACT complex

The histone chaperone FACT (facilitates chromatin transcription) has initially been identified as a transcription-related protein complex in yeast genetic screens (Malone et al., 1991; Rowley et al., 1991). The FACT complex consists of SPT16 and SSRP1 subunits in humans, while their two homologues in yeast are SPT16 and Pob3 respectively (Orphanides et al., 1999). However, yeast FACT also includes a third subunit, Nhp6, which does not form a stable ternary complex with SPT16 and Pob3, but is important for FACT recruitment and proper function (Formosa et al., 2001). In fact, the Nhp6 and Pob3 are fused into a single polypeptide as SSRP1 in metazoan. *In vitro*, FACT can stimulate transcription elongation through the nucleosome (Orphanides et al., 1998), while *in vivo* FACT is co-localized with RNA polymerase II (pol II) (Saunders et al., 2003). FACT is involved in facilitating the traverse of nucleosome by RNA polymerase, but it is also shown to regulate DNA replication and even DNA repair (Reinberg and Sims, 2006) (Keller et al., 2001; Yeeles et al., 2017). FACT is also shown to associate with the MCM helicase complex (Gambus et al., 2006) and DNA polymerase alpha (Foltman et al., 2013), and is involved in increasing the DNA accessibility during DNA polymerase traverse (Foltman et al., 2013). It plays redundant role with CAF-1 in replication-coupled nucleosome assembly (Tan et al., 2006; Yang et al., 2016a).

FACT is an abundant nucleus protein, which could account for one FACT complex per five nucleosomes in yeast (McCullough et al., 2015). FACT can bind histones H3-H4 and H2A-H2B simultaneously, making this complex a unique histone chaperone with the potential to organize nucleosome structure for RNA polymerase traverse (Tsunaka et al., 2016). Recent findings illuminate the mechanism of FACT in organizing nucleosome structure by destabilizing H2A-H2B dimers from the nucleosome, while maintaining the $(H3\text{-}H4)_2$ tetramer in a manner that allows for RNA polymerase traverse without splitting two H3-H4 dimers (Hsieh et al., 2013; Tsunaka et al., 2016). Since FACT has been shown to facilitate DNA replication as well, this epigenetically conservative manner during transcription might correlate with the conservative parental histone segregation during DNA replication. Indeed, recent studies suggest that FACT facilitates MCM helicase progress in a chromatin context as well (Kurat et al., 2017), but whether FACT is directly involved in histone deposition after the replication machinery remains unknown.

1.4.4 Rtt106

Rtt106 is an H3-H4 specific histone chaperone found in yeast, but no mammalian homologues have been demonstrated (Huang et al., 2005). Rtt106 is important for heterochromatin silencing in budding yeast. It acts synergistically with the other replication-coupled histone chaperone CAF-1, suggesting Rtt106 plays redundant roles in maintaining silent heterochromatin (Huang et al., 2005; Huang et al., 2007). The middle region of Rtt106 contains a tandem pleckstrin homology domain (PH domain) that binds to H3-H4, similar to the one found in FACT subunit SPT16 (Liu et al., 2010; Tsunaka et al., 2016), but it is unclear if Rtt106 binds to H3-H4 in a similar way as SPT16. RTT106 prefers H3-H4 binding, and recognizes H3K56ac (Su et al., 2012), a newly synthesized histone marker in budding yeast, suggesting Rtt106 is involved in new histone deposition during DNA replication. The middle tandem PH domain also can bind to DNA through a distinctive site from histone binding site (Liu et al., 2010). Both the histone

binding and DNA binding activity of Rtt106 are required for efficient heterochromatin silencing in a cooperative manner with CAF-1 (Liu et al., 2010). Rtt106 exists with an elongated conformation in solution, but it can dimerize through its N terminal domain (Fazly et al., 2012). The dimerization of Rtt106 is important for its binding to histone H3-H4 in a $(H3\text{-}H4)_2$ tetramer form, suggesting that two Rtt106 molecules are involved in tetramer deposition (Fazly et al., 2012; Su et al., 2012). Rtt106 also contains a 50 amino acids long acidic stretch at its C terminal end which is believed to neutralize the histone charges (Liu et al., 2010). Rtt106 has been shown to assemble nucleosomes, as tested with the supercoiling assay (Huang et al., 2005), but the mechanism of how Rtt106 delivers histones H3-H4 to DNA remains elusive.

1.4.5 CAF-1

CAF-1 is known as a chromatin assembly factor following DNA replication. More detailed introduction of CAF-1 is found in the next section.

1.5 Chromatin assembly factor-1

CAF-1 was originally identified as a nuclear factor involved in the assembly of chromatin during Simian virus 40 (SV40) origin based replication in human cell extracts (Smith and Stillman, 1989; Stillman, 1986). Human CAF-1, initially isolated from human cell lines, is a complex of three subunits on SDS-PAGE, with their bands corresponding to 150, 60, and 48 kDa, and they were thus named P150, P60 and P48, respectively (Smith and Stillman, 1989) (Table 1.1). Later on, CAF-1 was found to be conserved from yeast to human (Kaya et al., 2001; Smith and Stillman, 1989; Song et al., 2007; Takami et al., 2007; Tyler et al., 1999), yet the protein sizes are diverse among different species (Table 1.1).

CAF-1 is localized to replication sites in S phase through its interaction with PCNA, and associates with newly synthesized H3-H4 (Kaufman et al., 1995; Smith and Stillman, 1991a; Verreault et al., 1996) (Krude, 1995; Shibahara and Stillman, 1999). Therefore, CAF-1 acts as a histone chaperone that directly deposits newly synthesized histone H3-H4 onto DNA

Table 1.1 CAF-1 is a trimeric complex in all species. The names of the individual subunits in the various organisms are listed here.

species / subunits	*H. sapiens*	*S. cerevisiae*	*D. melanogaster*	*X. laevis*	*C. elegans*	*Arabidopsis*
Large	p150	Rlf2/Cac1	p180	p150	Chaf1	Fas1
Middle	p60	Cac2	p105/p75	p60	Chaf2	Fas2
Small	p48/RbAp48/RBBP4	Msi1/Cac3	p55/NURF-55	p48/RbAp48/RBBP4	Rba1	MSI1

(Kaufman et al., 1995). Recent studies also indicate that CAF-1 safeguards cell identity and is crucial for epigenetic maintenance during embryo development (Cheloufi et al., 2015; Houlard et al., 2006; Nabatiyan and Krude, 2004; Nakano et al., 2011).

CAF-1 is essential for the development and life of multi-cellular organisms (Cheloufi et al., 2015; Nakano et al., 2011; Song et al., 2007; Yu et al., 2015), except for in plants, in which case deletion of CAF-1 only affects development issue in shoot apical and root meristem (Kaya et al., 2001; Ono et al., 2006). CAF-1 is not essential for survival of yeast, however, CAF-1 depletion makes the yeast more sensitive to UV-damage, and knockdown of CAF-1 in yeast causes aberrant transcription and defective sub-telomeric scilencing (Kaufman et al., 1997; Monson et al., 1997).

1.5.1 CAF-1 function in DNA replication

As mentioned above, CAF-1 is recruited to the replication foci by interacting with PCNA for new histone deposition, and thus is required for proper chromatin restoration after DNA replication. CAF-1 expression is highly coordinated with the cell cycle, and it has been shown that p150 expression is partly regulated at the mRNA level in a cell cycle-dependent manner, and accumulates during S phase but fades away quickly when cells enter into quiescence (Polo et al., 2004), consistent with CAF-1 function in nucleosome assembly during DNA replication. RNAi-mediated knockdown of CAF-1 in human cells decreases the nucleosomes on newly synthesized DNA and prevents cells from progression through S-phase, with activation of the checkpoint for DNA replication defect (Hoek and Stillman, 2003; Nabatiyan and Krude, 2004; Ye et al., 2003).

1.5.2 CAF-1 function in DNA repair

DNA is constantly damaged in the cell when exposed to radiation or mutagenic chemicals. Defects in proper DNA repair would result in increased mutational rate and genome instability, and cause diseases such as cancer. Therefore efficient repair of damaged DNA is crucial for the

integrity of the genome and wellbeing of the cells. UV-induced DNA damage repair occurs simultaneously with chromatin assembly, and CAF-1 is involved in proper chromatin formation after DNA repair (Gaillard et al., 1996). Moreover, CAF-1 expression has been shown to be up-regulated in quiescent cells upon induction of DNA damage, and CAF-1 is recruited to DNA damage sites along with PCNA for efficient repair (Green and Almouzni, 2003; Nabatiyan et al., 2006).

As CAF-1 functions along with PCNA and DNA synthesis, CAF-1 is believed to be mainly involved in DNA-synthesis dependent DNA repair. There are two major DNA repair pathways that require DNA synthesis during repair process: NER (nucleotide excision repair) and HR (homologous recombination). However, the exact roles of CAF-1 in these events requires further investigations. It has been shown that CAF-1 p60 is hyperphosphorylated when recruited to UV-induced DNA damage sites (Martini et al., 1998). But it is unclear whether hyperphosphorylation regulates CAF-1 recruitment itself (Keller and Krude, 2000). Nevertheless, CAF-1 is thought to be required for re-packaging of chromatin after damage repair, by delivering new histone H3-H4 in a similar way to DNA replication (Polo et al., 2006).

1.5.3 CAF-1 function in epigenetic maintenance

CAF-1 also functions in proper heterochromatin maintenance through cell division. First, CAF-1, as a chromatin assembly factor, is localized to the replication sites in heterochromatin region, and is required for the replication of heterochromatin structure to prevent histone loss (Krude, 1995); second, CAF-1 interacts with HP-1 through MIR (MDG4 interacting region) on p150, to recruit HP-1 to maintain heterochromatin structure during replication (Quivy et al., 2008). Third, CAF-1 interacts with histone methyltransferase SETDB1 and Methyl-CpG binding protein MBD1 (Sarraf and Stancheva, 2004), suggesting that CAF-1 might coordinate concurrent repressive modifications on chromatin after heterochromatin replication. Lastly, CAF-1 is shown to

contribute to building functional kinetochores at the centromere (Sharp et al., 2002), potentially by maintaining a stable centromere structure after replication.

In yeast *S. cerevisiae*, knockdown of CAF-1 causes aberrant transcription and defective sub-telomeric silencing (Huang et al., 2007; Kaufman et al., 1997; Monson et al., 1997). Sir proteins, which are important for heterochromatin maintenance in budding yeast, are mislocalized when CAF-1 and Rtt106 are depleted. These data suggest that CAF-1 is involved in proper heterochromatin maintenance (Huang et al., 2007). Consistent with this, CAF-1 knockdown in human cells is associated with a decrease in somatic heterochromatin domains (Cheloufi et al., 2015).

1.5.4 Other CAF-1 functions

Besides the well-known histone chaperone function of CAF-1, multiple studies also suggest that CAF-1 is involved in other processes. For example, outside of S phase, CAF-1 has been shown to regulate the transcription directionality by suppressing noncoding transcription in a chromatin-based manner (Marquardt et al., 2014). In embryonic stem cells, p150 and p60 (but not p48) are recruited to and contribute to silencing of the expression of exogenous proviruses and endogenous retroviruses (ERVs) through epigenetic co-factors, a key step for normal embryonic development (Yang et al., 2015). Moreover, the N terminus of p150 in human has been shown to regulate the localization of Ki-67, a fundamental component of the perichromosomal layer that shields condensed chromosomes during mitosis (Matheson and Kaufman, 2016; Smith et al., 2014), but whether this function is dependent on the integrity of CAF-1 remains unknown.

1.5.5 Current knowledges of CAF-1 structure

1.5.5.1 CAF-1 is a heterotrimeric protein complex

CAF-1 is a heterotrimer consisting of three subunits conserved from yeast (Cac1, Cac2 and Cac3) to human (p150, p60 and p48) (Figure 1.5) (Smith and Stillman, 1989; Song et al., 2007;

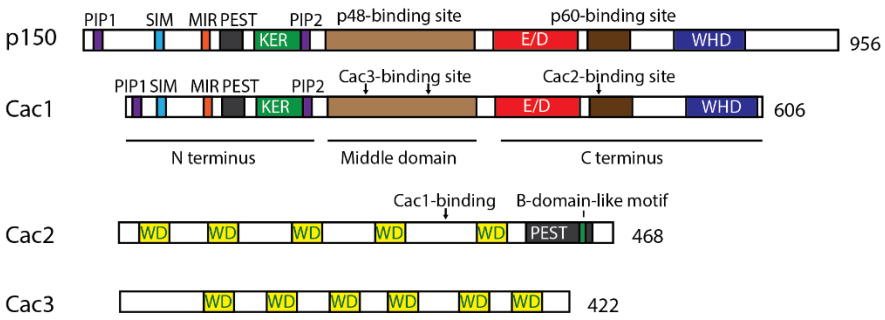

Figure 1.5 Schematic view of human p150 and three subunits of yeast CAF-1. PIP: PCNA interacting peptide. SIM: sumo-interacting motif. MIR: MDG4 (Mod)-interacting region. PEST: A region possible for regulation of protein degradation (Rogers et al., 1986). E/D: acidic region rich of glutamic acid and aspartic acid. WHD: winged helix domain. WD: WD40 domain. The potential binding sites are listed based on previous studies as well as studies in this disertation (Kaufman et al., 1995).

Takami et al., 2007; Tyler et al., 1999). Table 1.1 lists conserved CAF-1 subunits in different species. There was little structural information about CAF-1 complex when I started this project, except for the crystal structures of the small subunit of fly and human CAF-1 (Alqarni et al., 2014; Murzina et al., 2008; Song et al., 2008). In 2016, a low resolution EM structure of yeast CAF-1 complex was resolved but no details are unveiled in terms of subunits organization (Kim et al., 2016).

The large subunit

Mammalian CAF-1 large subunit p150 has been shown to interact with numerous proteins, including afore-mentioned PCNA, HP-1, and MBD1. The large subunit of CAF-1 is less conserved compared to the other two subunits, and is structurally predicted to be flexible throughout most regions. The N-terminal region of Cac1 is predicted to be largely unfolded, and therefore is not characterizable from EM (Kim et al., 2016). This region is not required for CAF-1 histone binding and nucleosome assembly in purified systems (Kaufman et al., 1995), but it contains several functional motifs, suggesting a regulatory function for spatiotemporally proper CAF-1 - mediated nucleosome assembly and epigenetic maintenance.

The N-terminal region of Cac1 contains two PCNA interacting peptide sequences based on the conservative comparison to classic PCNA binding motifs. PIP1 (PCNA Interacting Peptide) has stronger affinity than PIP2, but PIP2 rather than PIP1 is indispensable for replication-coupled chromatin assembly *in vitro* (Rolef Ben-Shahar et al., 2009), consistent with the fact that PIP2 is more evolutionally conserved (Dohke et al., 2008). Next to the PIP2 is a region full of charged residues (named KER region from here on); this region is predicted to be in a coiled-coil structure and shown to bind to DNA (Sauer et al., 2017). The N-terminal region also has a Mod-1 interacting region (MIR) which associates with HP-1, and this interaction guarantees the recruitment of HP-1 to replicative sites (Murzina et al., 1999; Quivy et al., 2004). Overall, the N terminus of Cac1, the large subunit of CAF-1, is largely flexible and less conserved, and

contains several functional domains that regulate the protein lifetime, specific recruitment and interplay with multiple proteins for fine tuning CAF-1 function. Notably, deletion of the N terminus from PIP does not affect CAF-1 assembly (Kaufman et al., 1995).

The C-terminus of p150 in human CAF-1 has been shown to contain essential domains for its nucleosome assembly activity (Kaufman et al., 1995). Around the center of the CAF-1 large subunit, there is an acidic ED-rich region, a common feature found in many other chaperones (Warren and Shechter, 2017), which is proposed to be important for histone binding (Kaufman et al., 1995; Liu et al., 2016).

The large subunit from human CAF-1 also has been proposed to contain a dimerization domain at the C terminus of the ED-rich region (Quivy et al., 2001), which is important for proper chromatin establishment in Xenopus embryo development. However, this dimerization interaction is very weak, without clear *in vitro* evidence (Quivy et al., 2001).

At the very end of the large subunit Cac1, there is a winged helix domain (WHD), whose structure has been recently solved by crystallography (Zhang et al., 2016b). This is the first structure of a domain from the large subunit of CAF-1. The WHD is shown to bind DNA with low μM range, but without any sequence preference as tested (Zhang et al., 2016b). The WHD coordinates with the PIP domain for CAF-1 retention at the replication foci (Zhang et al., 2016b).

The middle subunit

The middle subunit of CAF-1 contains 5-7 WD repeats, but none of the CAF-1 middle subunits from any species has been structurally characterized. WD40 repeat containing proteins form canonical β barrel structures, which are believed to be adaptor proteins involved in protein-protein interaction platform (Xu and Min, 2011), and they have been shown to bind to the H3 tail through the top of β-barrel structure, and bind to H4 α1 helix through a pocket at the side of

the barrel structure (Suganuma et al., 2008). The middle subunit of CAF-1 forms WD40 propellers structures (Alqarni et al., 2014; Song et al., 2008).

The middle subunit also contains a B-like domain (B domain is a short peptide found in HIRA to be associated with Asf1) at the C terminus that has been shown to associate with histone chaperone Asf1 (Kirov et al., 1998; Malay et al., 2008; Tang et al., 2006; Zhang et al., 2005b), suggesting that the middle subunit functions as a regulator to receive histone H3-H4 from Asf1. The middle subunit of CAF-1 resembles the HIRA subunit of HIRA complex, which also contains a WD40 propeller structure followed up with a B-domain. Previously studies suggests that the human CAF-1 middle subunit binds to the C terminus of p150, without any clear identification of which region p60 is binding to.

The middle subunit is mainly functioning in the CAF-1 complex, with no published data supporting a role in other complexes. In support of this, RNAi-induced knockdown of p150 in human cells caused concurrent depletion of p60, indicating p60 is not stable and is thus subject to degradation when isolated *in vivo* (Hoek and Stillman, 2003).

Human p60 is a target for phosphorylation, and it contains several consensus sites for protein kinase C, Casein kinase 2 and tyrosine kinase (Martini et al., 1998; Smith and Stillman, 1991a). The phosphorylation status changes according to the cell cycle transitions or during induced DNA damage (Marheineke and Krude, 1998; Smith and Stillman, 1991a) (Martini et al., 1998). Part of p60 is dephosphorylated during the S phase, and is more localized to chromatin, however it is not clear which positions on p60 gets dephosphorylated and whether p60 phosphorylation status has a role in CAF-1 recruitment onto chromatin (Marheineke and Krude, 1998). In G2 phase, phosphorylated p60 is totally dissociated from chromatin (Marheineke and Krude, 1998), probably indicating that the cell cycle dependent p60 phosphorylation status might regulate the association of CAF-1 with chromatin. In contrast, UV-induced DNA damage triggers phosphorylation of p60 in Hela cells, and results in accumulation of phosphorylated p60

onto chromatin, presumably the sites of DNA repair. Besides that, CAF-1 complex purified from synchronized G2 phase cells are compromised during complementary DNA strand synthesis (Marheineke and Krude, 1998). However, the exact roles of p60 phosphorylation in regulating CAF-1 recruitment and nucleosome assembly activity requires further investigation.

The small subunit

Similar to the middle subunit, the small subunit also contains several WD repeats, and forms canonical β-barrel structures. So far, only the small subunit of CAF-1 from different species has been structurally characterized (Alqarni et al., 2014; Murzina et al., 2008; Song et al., 2008). The small subunit is the most abundant protein compared to the other two subunits in animals, and actually over 80% of this protein is associated with other complexes such as the remodeling complex NURF, the histone deacetylase complex NuRD and the Polycomb respressive complex 2 (PRC2) (Barak et al., 2003; Nowak et al., 2011; Zhang et al., 1999). However, in yeast, due to the lack of these complexes, the amount of Cac3 is relatively less than the other two subunits (Ghaemmaghami et al., 2003). The small subunit of CAF-1 has been shown to bind both H3 and H4 *in vitro* (Nowak et al., 2011) (Schmitges et al., 2011). Interestingly, the small subunit of CAF-1 in humans and flies binds to the α1 helix of H4 through a groove on the side of beta-barrel structure (Murzina et al., 2008; Song et al., 2008), while the top of beta-barrel binds to the N terminal tail of H3 (Nowak et al., 2011; Schmitges et al., 2011). Thus, the conformation of H3-H4 when bound to the small subunit of CAF-1 is not compatible with their conformations within the nucleosome. Therefore, it is still unclear how the small subunit contributes CAF-1 for its histone binding activity or nucleosome assembly activity.

1.5.6 Summary of CAF-1

As discussed above, CAF-1 is definitely important for proper chromatin restoration after replication, and for the preservation of epigenetic states in the cells. More importantly, as a histone chaperone, CAF-1 fulfils its functions mainly through its chaperone activity, but also

coordinates with other factors. However, the biochemical and biophysical properties of CAF-1 to histone, DNA, nucleosome and chromatin were largely unknown when I started this project.

1.6 Overview of dissertation

Although CAF-1 was first identified over two decades ago, the structure of this complex and its mechanism remains elusive. It has been proposed before that the small subunit of CAF-1 or/and the acidic region on Cac1 is important for histone binding, however the histone binding property of the small subunit in the context of CAF-1 is not clear, and the function of the middle subunit has not been demonstrated before. Moreover, the molecular mechanism of nucleosome assembly by CAF-1 is unknown. Lastly, the contributions of H3-H4 tails to CAF1 mediated nucleosome assembly are still under debate. Therefore, there are two major aims in my project: Aim 1: Characterization of the association between subunits of the CAF-1 complex and histone H3-H4; Aim 2: Characterization of the mechanisms of nucleosome assembly by CAF-1.

In Chapter 2, I describe the expression and purification of recombinant CAF-1 proteins from insect cells with dramatically improved efficiency and yield. In collaboration with Dr. Francesca Mattiroli, we performed HX-MS (hydrogen-deuterium exchange coupled to mass spectral analysis), and successfully mapped the peptides involved in CAF-1 complex assembly. Based on these interface information, I cloned and purified sub-complexes of CAF-1, which could be used to identify and verify important regions/domains/subunits for histone binding and nucleosome assembly.

In Chapter 3, I developed and optimized a nucleosome assembly and quantification assay (NAQ assay) to track the nucleosome assembly activities of CAF-1, which is more quantifiable compared to classical supercoiling experiments. This assay allows us to screen CAF-1 mutants that have defects in nucleosome assembly. We found that the WH domain plays an essential role in coordinated DNA interaction and joining of two CAF-1 complexes for proper tetrasome assembly, a key process for nucleosome assembly. Interestingly, further characterization

indicates that the WHD is auto-inhibited in CAF-1, suggesting a complicated regulatory mechanism among CAF-1 domains for efficient histone H3-H4 deposition. Moreover, in this chapter, I used chemically crosslinked $(H3\text{-}H4)_2$ tetramer and constitutive dimer mutant H3-H4 (DMH3-H4) to perform fluorescence-based affinity and stoichiometry measurement for CAF-1 histone binding. Along with HDX-MS experiment, we mapped the CAF-1 binding interfaces on histones.

In chapter 4, we re-designed the constitutive dimer mutant H3-H4 for more stable and consistent experiments. I compared the nucleosome formation ability between this DMH3-H4 and wild type H3-H4, and found that DMH3-H4 could not be assembled into nucleosomes by histone chaperones. This indicates that functional tetramerization of H3-H4 is a prerequisite for actual nucleosome assembly *in vivo*. Nevertheless, this DMH3-H4 could be reconstituted into nucleosomes by salt dialysis or salt dilution. When comparing the thermal stability between WT nucleosome and DM nucleosome, I found that the DM nucleosome completely dissociates without H3-H4-DNA complex left, while the WT nucleosome will dissociate to form tetrasome or hexasome when H2A-H2B escaped. Finally using different DNA sequences, I found that both histone dimer interaction and histone-DNA interaction contribute to making nucleosome a stable complex.

CHAPTER 2: THE CAC2 SUBUNIT IS ESSENTIAL FOR PRODUCTIVE HISTONE BINDING AND NUCLEOSOME ASSEMBLY IN CAF-1[1]

Overview

Nucleosome assembly following DNA replication controls epigenome maintenance and genome integrity. Chromatin assembly factor 1 (CAF-1) is the histone chaperone responsible for histone (H3-H4)2 deposition following DNA synthesis. Structural and functional details for this chaperone complex and its interaction with histones are slowly emerging. Using hydrogen-deuterium exchange coupled to mass spectrometry, combined with *in vitro* and *in vivo* mutagenesis studies, we identified the regions involved in the direct interaction between the yeast CAF-1 subunits, and mapped the CAF-1 domains responsible for H3-H4 binding. The large subunit, Cac1 organizes the assembly of CAF-1. Strikingly, H3-H4 binding is mediated by a composite interface, shaped by Cac1-bound Cac2 and the Cac1 acidic region. Cac2 is indispensable for productive histone binding, while deletion of Cac3 has only moderate effects on H3-H4 binding and nucleosome assembly. These results define direct structural roles for

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[1] Declaration of contributions:

This chapter was published (Mattiroli et al., 2017a) as a collaboration between the following authors: Francesca Mattiroli*, Yajie Gu*, Jeremy L. Balsbaugh, Natalie G. Ahn & Karolin Luger. I chose to include this full manuscript into this chapter because the presented data has been peer-reviewed and the conclusions out of this manuscript partially fulfill the goal of my project. I am responsible for the cloning, expression and purification of full length CAF-1, tCAF-1, tCAF_Δ3, Cac2, HBM, tCAF_ MrΔ2, tCAF_CrΔ3 and the tCAF mutants used in Figure 2.4 *in vitro* pull-down experiments. I am responsible for the affinity measurements, tetrasome assembly and nucleosome assembly activities of all the mutants in table 2.1. The HX-MS was performed in full collaboration with Francesca Mattiroli. I performed studies shown in Figures 2.1 e; 2.4 a, b, d; 2.5 a, c, d; 2.6 b, c, d. Dr. Mattiroli and I were equally responsible for interpreting the data.

yeast CAF-1 subunits and uncover a previously unknown critical function of the middle subunit in CAF-1.

2.1 Introduction

Nucleosomes are the primary units of chromatin responsible for the organization of DNA and storage of epigenetic information. The interaction between positively charged histones and negatively charged DNA must be tightly regulated *in vivo* to avoid the formation of non-specific histone•DNA complexes (Gurard-Levin et al., 2014). Histone chaperones guard histones by shielding their charges and controlling their deposition onto DNA(Elsasser and D'Arcy, 2013). This places histone chaperones at a central position in all pathways that require access to the genome.

During DNA replication, the concerted action of a number of histone chaperones coordinates the recycling of parental histones and the deposition of newly-synthesized histones to maintain nucleosome density and epigenetic information (Alabert and Groth, 2012). Chromatin Assembly factor 1 (CAF-1) is a heterotrimeric histone chaperone that mediates the final step in this cascade by assembling nucleosomes following DNA synthesis (Almouzni and Mechali, 1988; Kaufman et al., 1995; Kaufman et al., 1997; Smith and Stillman, 1989; Verreault et al., 1996). CAF-1 directly interacts with the processivity factor for DNA polymerases, PCNA, which localizes to sites of DNA synthesis during replication and repair (Gaillard et al., 1996; Martini et al., 1998; Moggs et al., 2000; Rolef Ben-Shahar et al., 2009; Shibahara and Stillman, 1999; Zhang et al., 2000). The unique role of CAF-1 in chromatin assembly following DNA synthesis makes it essential for cell survival in multicellular organisms (Cheloufi et al., 2015; Houlard et al., 2006; Nakano et al., 2011; Song et al., 2007). In yeast, deletion of either CAF-1 subunit results in cell viability but affects transcriptional silencing and DNA damage sensitivity (Kaufman et al., 1997).

The structural organization of the CAF-1 complex is only beginning to emerge(Kim et al., 2016; Liu et al., 2016), and the direct contribution of each subunit to CAF-1 functions remain unclear. Histone binding by CAF-1 has been attributed to the Cac1 acidic region(Kaufman et al., 1995; Liu et al., 2016), a ~50 amino acids stretch, primarily composed of Glu and Asp residues. Other studies have attributed this function to the small subunit (Cac3 in yeast), as the human and fly homologues bind histones H3 and H4 when tested in isolation(Nowak et al., 2011; Schmitges et al., 2011; Song et al., 2008; Zhang et al., 2013). Notably, this subunit is shared with other chromatin-related complexes, such as histone deacetylase complexes, NuRD, NURF and PRC2(Barak et al., 2003; Ciferri et al., 2012; Johnston et al., 2001; Millard et al., 2016; Parthun et al., 1996). Nonetheless, the direct contribution of this subunit to histone binding in the context of the full CAF-1 complex has not yet been tested. In cells, histones are handed off to CAF-1 by the histone chaperone Asf1, which directly interacts with the C-terminal region of the CAF-1 middle subunit(Malay et al., 2008; Mello et al., 2002; Tang et al., 2006; Tyler et al., 2001), yet beyond that, the role of the middle subunit for CAF-1 activity remain elusive.

Here we focus on the yeast CAF-1 complex and identify the regions that mediate complex formation between the three CAF-1 subunits (Cac1, Cac2 and Cac3). We show that these interfaces are relevant for CAF-1 function *in vivo*. We find that when bound to Cac1, Cac2 is indispensable for productive interaction with the histones, thus their deposition. The Cac1 acidic region contributes to histone binding and together, these shape a composite interface on CAF-1 for H3-H4 binding.

2.2 Results

2.2.1 CAF-1 is an elongated complex

Recombinant full-length (FL) yeast CAF-1 complex was expressed in SF21 cells using the MultiBac system (Bieniossek et al., 2008). We used small angle X-ray scattering in line with size exclusion chromatography (SEC-SAXS) to investigate the architecture of the complex (Figure

2.1a-c). Previous data showed that the three CAF-1 subunits, Cac1, Cac2 and Cac3, assemble in a 1:1:1 stoichiometry (Liu et al., 2012), which was confirmed by our analysis with size exclusion chromatography in line with multi-angle light scattering (SEC-MALS) (Figure 2.1d). The SAXS probability function (P(r)) displays a bimodal distribution indicative of two structural units connected by a less organized linker, with an extended maximum distance for the entire complex of 250-300 Å (Figure 2.1b). This can be visualized in the ab initio model generated from the SEC-SAXS data (Figure 2.1c). An extended conformation of the complex is also suggested by SEC-MALS, where CAF-1 elutes from the column much earlier than expected for a globular complex with similar molecular weight (Figure 2.1d). Based on their expected size, we speculate that the two lobes in the ab initio model identify the location of the two WD40 subunits, Cac2 and Cac3 (Figure 2.1c, arrows), while Cac1 is likely responsible for the elongated shape.

2.2.2 Cac1 mediates CAF-1 complex assembly

Previous work showed that the middle subunit of CAF-1 interacts with the C-terminal region of the large subunit (Cac2 and Cac1 respectively in yeast) (Kaufman et al., 1995). We determined through proteolysis experiments that Cac3 interacts with the central portion of Cac1 (from residue 233; Figure 2.2). Based on this information, we engineered a Cac1 fragment starting at residue 233 and extending to the very C-terminal amino acid 606 (named tCac1 for truncated Cac1), to obtain a minimal Cac1 construct still able to form a complex with the other CAF-1 subunits. Indeed, tCac1 formed a trimeric complex together with Cac2 and Cac3, which we name tCAF-1. This complex maintains a 1:1:1 stoichiometry (Figure 2.1d) and binds histones H3-H4 with the same affinity as FL CAF-1 (Figure 2.1e and Table 2.1), demonstrating that tCAF-1 retains the molecular requirements for CAF-1 trimer assembly and histone binding.

To identify the peptides involved in mediating the interactions between CAF-1 subunits, we applied hydrogen-deuterium exchange coupled to mass spectrometry (HX-MS) to the tCAF-1

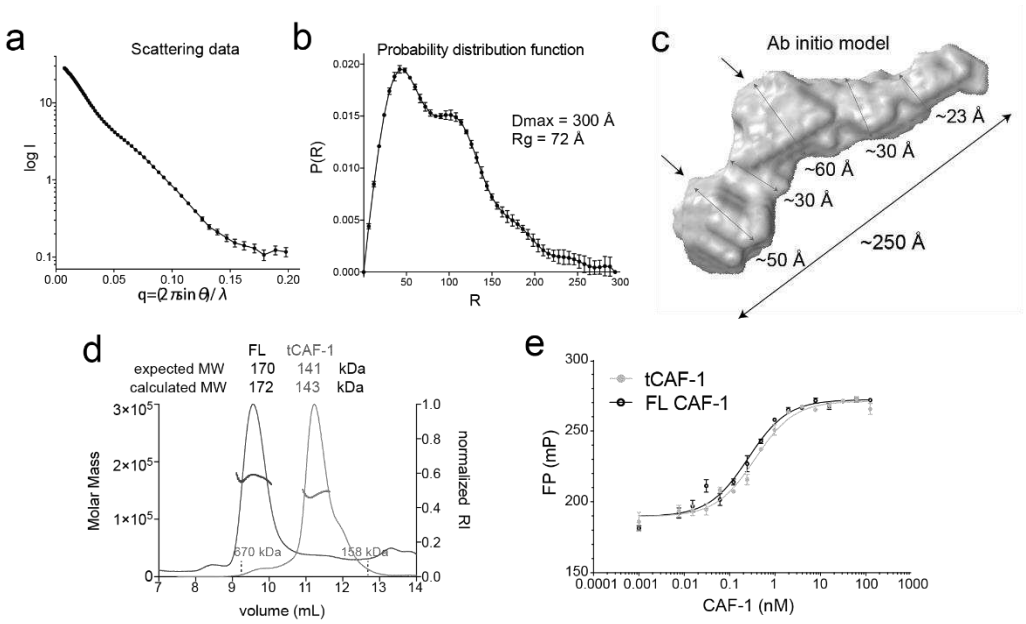


Figure 2.1 CAF-1 is an elongated complex

a) SAXS scattering data for the full length CAF-1 complex. b) Probability distribution function from the SAXS data. c) Calculated ab initio model from SAXS data. Arrows indicate putative locations of Cac2 and Cac3, respectively, also informed by mass spectrometry. d) SEC-MALS traces of FL CAF-1 and tCAF-1. The protein/DNA elution traces (refractive index, RI) refer to the right y axis, the measured molar mass data points refer to the left y axis. e) Fluorescence polarization measurement of the affinity of FL CAF-1 and tCAF-1 for Alexa488-labeled H3-H4.

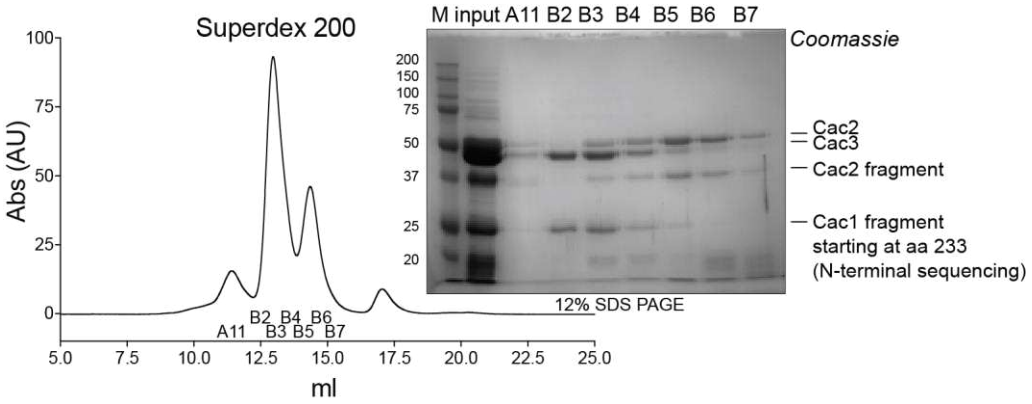


Figure 2.2 Limited degradation of FL CAF-1 analyzed by gel filtration and MS.

Size-exclusion chromatography trace (Abs 280 nm) of a degrading preparation of FL CAF-1. The injected sample and highlighted fractions are analyzed on a 12% SDS PAGE.

complex and its sub-complexes lacking either the middle subunit Cac2 (tCAF_Δ2) or the small subunit Cac3 (tCAF_Δ3), as well as to the Cac2 and Cac3 subunits in isolation. Notably, all of these complexes could be purified using standard protocols and eluted as well-behaved monodisperse complexes of the expected size and stoichiometry in gel filtration (Figure 2.3 a). HX-MS can be used to identify binding interfaces and conformational changes in protein complexes due to the specific protection from exposure to deuterated solvents as a consequence of protein-protein interactions(Englander, 2006). From the HX-MS experiments, we obtained information on about 110-120 peptides from each subunit per sample, with a protein coverage of 90% for Cac1, 94% for Cac2 and 95% for Cac3. For comparative purposes we then focused only on peptides that were present in all samples, bringing the protein coverage to 82% for Cac1, 89% for Cac2 and 85% for Cac3.

To identify the Cac1 regions responsible for Cac3 and Cac2 binding, we compared deuteron uptake of Cac1 peptides within the entire tCAF-1 complex to uptake in the tCAF_Δ3 or tCAF_Δ2 complex, respectively (Figure 2.3 b-c). Strikingly, upon omission of either subunit, a clear increase in deuteron uptake is seen in distinct regions of tCac1. Cac3 binding to Cac1 results in protection on residues 275-390 in Cac1, while Cac2 binding protects a much shorter region on Cac1 (aa 436-475) (Figure 2.3 b-c). These two regions are separated by the Cac1 acidic domain (aa 383-436), for which we observed low coverage in our HX-MS analysis (Figure 2.3b). Based on these results, we designed charge neutralization and reversal mutations that could abrogate individual CAF-1 subunit interactions *in vitro*. We found that two peptides on Cac1 (peptide 280-286 and 344-349) contact Cac3, and either site is sufficient to support Cac1 interaction with Cac3, as shown by pulldown assays from insect cell lysates expressing either wild type or mutant proteins (Figure 2.4a). Cac1-Cac3 interaction was abolished by mutating both regions (tCac1_3AA and tCac1_3AD), but not by the mutation on a single peptide (tCac1_3a and tCac1_3bA or tCac1_3bD) (Figure 2.4a). With respect to Cac2, a charged

Table 2.1: H3-H4 binding affinity and activity of tCAF-1 subcomplexes.

Complex	**H3-H4 affinity - Kd (95% confidence intervals)**	**Tetrasome assembly**	**Nucleosome assembly**
FL CAF-1	0.3 nM (0.27 to 0.45)	++	++
tCAF-1	0.5 nM (0.30 to 0.65)	++	++
tCAF_Δ3	1.3 nM (0.91 to 1.79)	+	+
tCAF_Δ2	25 nM (12.1 to 37)	-	-
Cac2	> 1 µM	-	-
Cac3	77 nM (64.9 to 89.4)	-	-
HBM	1.9 nM (1.32 to 2.68)	N.D.	N.D.
tCAF_CrΔ3	> 1 µM	N.D.	N.D.
tCAF_ MrΔ2	> 1 µM	N.D.	N.D.
tCAF_ Δac	60 nM (41.9 to 78.8)	+	+
tCAF_ Nac	8 nM (6.16 to 9.29)	+	+

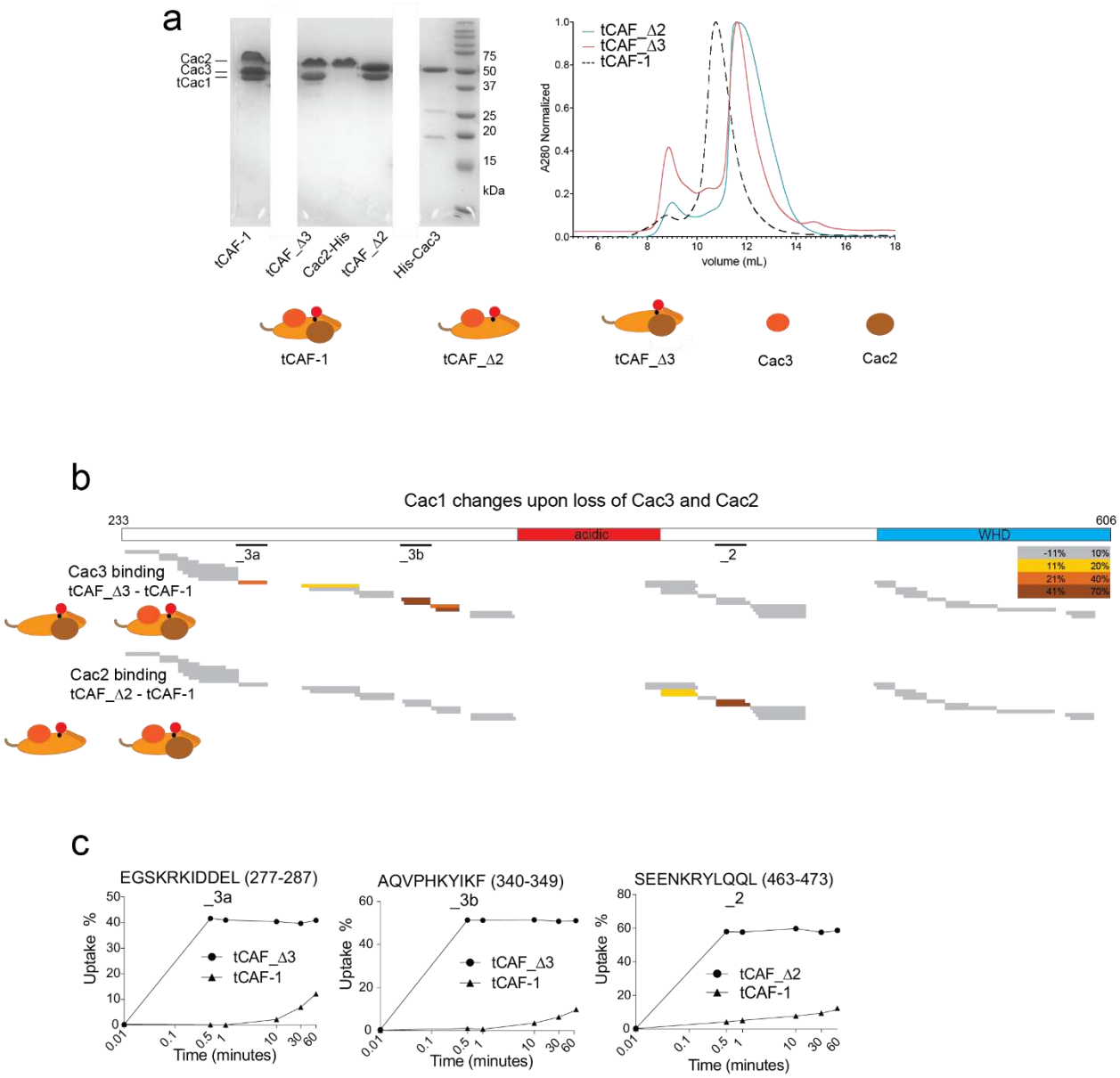


Figure 2.3 Cac1 mediates CAF-1 complex formation

a) Validation of the quality of tCAF-1, subcomplexes and subunits used in HX-MS. Left panel shows samples analyzed on 15% SDS-PAGE; right panel shows gel filtration traces of tCAF-1 complexes. A280 absorbance is normalized for comparison; bottom panel shows a schematic of different complexes. b) HX-MS heatmap of the differences in deuteron uptake at 60 minutes in Cac1, when Cac3 or Cac2 are omitted from the sample (comparison between tCAF_Δ3 or tCAF_Δ2 respectively). The difference was calculated as percent uptake in the subcomplex (unbound form) minus the percent uptake in tCAF-1 (bound form). c) Uptake plots of the Cac1 peptides mutated to test Cac2 and Cac3 binding.

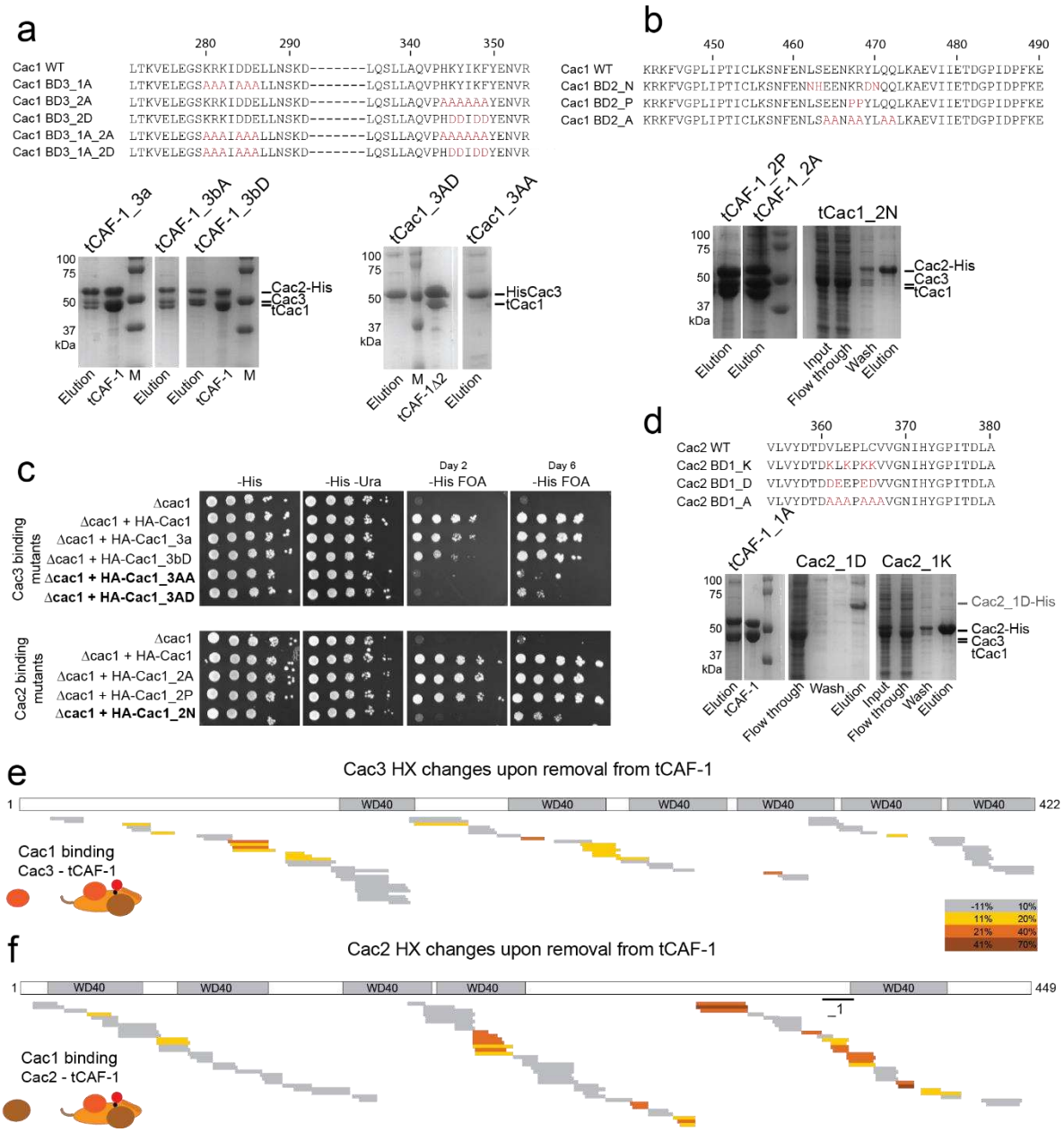


Figure 2.4 Cac1 mediates CAF-1 complex formation (continued)

a) Alignment of the Cac1 regions interacting with Cac3, and the mutants designed and analyzed in this study. SDS PAGE of the His-pulldown from insect cells using the Cac1_3a, Cac1_3b or the Cac1_3 mutants (mutated both on peptide a and b). Cac2-His was used as a bait in the left panels. His-Cac3 was used as a bait in the right panels (Cac2 was not present in these samples). b) Alignment of the Cac1 regions interacting with Cac2 and the mutants designed in this study. SDS PAGE of the His-pulldown from insect cells using the Cac1_2 mutant proteins. Cac2-His was used as a bait. c) Silencing assay performed with yeast strains expressing the Cac1 mutations at the _3a, _3b or _2 sites. Samples were spotted at 0-101-102-103-104 dilutions from a OD600=1 stock. d) Alignment of the Cac2 region interacting with Cac1 and the mutants designed. SDS PAGE of the His-pulldown from insect cells using the Cac2_1 mutants as bait. Supplementary File 1 contains all the HX uptake values for the experiments shown in

this figure. e) HX-MS heatmap of the differences in deuteron uptake at 60 minutes in Cac3, when bound in tCAF-1. The difference was calculated as percent uptake in the single subunit (unbound form) minus the percent uptake in tCAF-1 (bound form). WD40 domain mapping in the schematics is based on the Uniprot prediction. f) HX-MS heatmap of the differences in deuteron uptake at 60 minutes for Cac2 when it's removed from tCAF-1. The difference was calculated as in panel e but related to Cac2. WD40 domain mapping in the schematics is based on the Uniprot prediction. _1 depicts the peptide used for the mutagenesis studies shown in panel d.

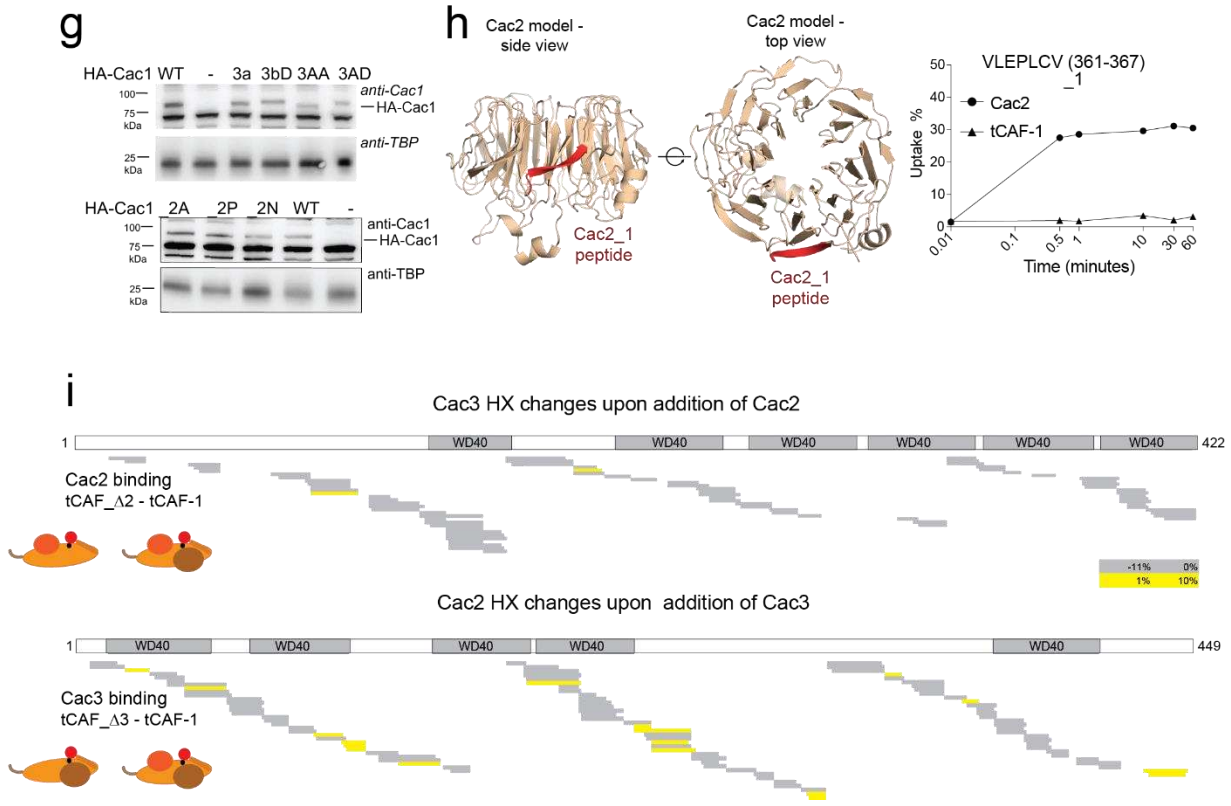


Figure 2.4 (continued)

g) Western blot of the lysate of yeast strains used for the spot assay shown in Figure 2d, to verify expression of the Cac1 constructs. TBP was used as loading control. Cells were grown in minimal media without histidine to an OD600= 0.8. h) Structural model of Cac2 (calculated by Phyre2). The Cac2_1 peptide which binds to Cac1 is highlighted in red. Uptake plot from the HX-MS data of the Cac2_1 peptide. i) HX-MS heatmap of the differences in deuteron uptake at 60 minutes in Cac3 (above) and Cac2 (below) measured when probing their direct interaction. The difference was calculated as percent uptake in the subcomplexes (unbound form) minus the percent uptake in tCAF-1 (bound form). WD40 domain mapping in the schematics is based on the Uniprot prediction. Supplementary File 1 (seen https://www.nature.com/articles/srep46274#s1) contains all the HX uptake values for the experiments shown in this figure.

mutation on Cac1 residues 463-475 (tCac1_2N) abolishes the interaction with Cac1, demonstrating the involvement of this region in binding Cac2 (Figure 2.4b).

We tested these Cac1 mutations *in vivo*, by monitoring the transcriptional state of a sub-telomeric reporter URA3 gene, which is dependent on CAF-1 integrity (Kaufman et al., 1997). In the absence of Cac1, growth on FOA-containing plates is inhibited due to the aberrant expression of the reporter gene, a phenotype that can be rescued by ectopic expression of the wild type FL CAC1 subunit (Figure 2.4c). Strikingly, Cac1 mutants that disrupt binding to Cac2 or Cac3 in the *in vitro* pull-down experiments, show an abnormal growth in this assay, consistent with aberrant CAF-1 functions (Figure 2.4c), while mutants that are still able to bind Cac2 and Cac3 *in vitro* have no phenotype and fully rescue the CAC1 deletion (Figure 2.4c). Normal levels of expression of all mutant proteins in yeast was demonstrated by immunoblot (Figure 2.4g). These data show that interfering with CAF-1 subunit interactions *in vivo* leads to dysfunctional CAF-1 complexes. Importantly, in these CAC1 mutant strains, the Cac2 and Cac3 protein levels are not directly affected, but rather the proteins are selectively removed from CAF-1. This implies that Cac3 still may function normally in the other complexes that it participates in (Barak et al., 2003; Ciferri et al., 2012; Johnston et al., 2001; Millard et al., 2016; Parthun et al., 1996).

Cac2 and Cac3, which are predicted to have a typical WD40 fold, both undergo extensive changes in HX when part of the tCAF-1 complex (Figure 2.4e-f), suggesting that their overall conformation is affected upon complex formation. To pinpoint the regions involved in direct binding we focused on peptides that show an almost complete protection (deuteron uptake close to 0%) in the bound form, similar to the peptides we have identified on Cac1 (Figure 2.3c). The complete lack of deuteration in the bound form for these peptides suggests a binding site that is comprised of essentially the entire observed amino acid sequence. We identified a number of peptides that filled these criteria in Cac2, but differences observed in Cac3 were

much smaller making it difficult to predict direct binding interfaces. We therefore designed mutations only on Cac2, based on the observed HX changes. Charged mutations of residues 361-367 (named Cac2_1) lead to loss of binding to tCac1, without affecting Cac2 expression (Figure 2.4d). Interestingly, in a homology model of Cac2, this region is predicted to be exposed (Figure 2.4h), supporting the notion that it may directly mediate binding to Cac1. Mutations at other peptides on Cac2 that show significant HX changes disrupted protein expression and hence could not be directly tested for their involvement into binding Cac1. These results support the idea that the Cac1-Cac2 interaction occurs between residues 462-472 on Cac1 and residues 361-367 on Cac2, primarily via hydrophobic interactions.

Only very subtle HX changes are detected when probing the Cac2-Cac3 interface within tCAF-1 (Figure 2.4i), suggesting that these two subunits may not contact each other when bound to Cac1.

Overall, using HX-MS combined with analyses of complex formation in yeast, we have identified regions on each subunit mediating direct interactions between Cac1, Cac2 and Cac3. We show that Cac1 mediates all key interactions that hold the CAF-1 trimer together, supporting previous *in vitro* reconstitutions and recent crosslinking data (Kaufman et al., 1995; Kim et al., 2016; Krawitz et al., 2002; Liu et al., 2016). We have identified mutations in the Cac1 and Cac2 subunit that do not affect expression or folding of the mutant subunits, yet disrupt complex formation and compromise CAF-1 functions *in vivo*, and therefore can be used to directly investigate CAF-1 subunit contributions in cells without the need of depleting the proteins in the nucleus. This is particularly important to study the role of Cac3 *in vivo*, because it also participates in other complexes outside of the CAF-1 complex.

2.2.3 Cac3 is dispensable for H3-H4 binding in CAF-1, while Cac2 is required

We next wanted to identify the CAF-1 regions involved in histone binding. To this end, we used the tCAF-1 subcomplexes and deletion constructs in H3-H4 binding assays. Cac3 in isolation is

able to bind H3-H4, as was observed for its metazoan homologues (Nowak et al., 2011; Schmitges et al., 2011; Song et al., 2008). However, its affinity for H3-H4 is over 50-fold weaker than that of the trimeric tCAF-1 complex (Figure 2.5a and Table 2.1). We therefore tested whether Cac3 was required for histone binding in the context of CAF-1. Strikingly, removal of Cac3 only had a minor effect on H3-H4 binding affinity, while the omission of Cac2 resulted in a 50-fold decrease of binding affinity, even though it has no affinity for histones when tested in isolation (Figure 2.5a and Table 2.1). This demonstrates that Cac2, together with Cac1, is important to shape the H3-H4 binding interface on CAF-1, with very little contribution from Cac3.

The contributions of Cac2, but not of Cac3, to H3-H4 binding are surprising in light of previous observations suggesting that Cac3 coordinates histone binding (Nowak et al., 2011; Schmitges et al., 2011; Song et al., 2008; Zhang et al., 2013). To further validate these findings, we investigated how these subunits contribute to histone binding using HX-MS. We decided to focus on HX changes in the histone proteins themselves to investigate how their accessibility to solvent is affected in the absence of Cac2 or Cac3, as analysis of the tCAF-1 subunits shows only subtle and distributed changes overall (Liu et al., 2016) (Mattiroli et al, related manuscript). We have shown that the trimeric tCAF-1 complex stabilizes the histone fold core of WTH3-H4, but does not interfere with the tetramerization of the histones, as no significant changes are seen in the C-terminal part of H3 (Mattiroli et al, related manuscript). Here we show, in line with binding affinity data reported in Figure 3a, that Cac3 removal appears to have a minimal effect on the protection pattern of histones H3-H4 (Figure 2.5b) when comparing the maximum deuteron uptake at 60 minutes for all histone peptides. In fact, the HX profiles of WTH3-H4 are very similar when bound to tCAF-1 or tCAF_Δ3, suggesting only a minor role, if any, for Cac3 in organizing H3-H4 binding in CAF-1. In contrast, in the absence of Cac2, we observe significant differences in the HX protection profile, compared to histone interactions with trimeric tCAF-1 (Figure 2.5b). In this complex (tCAF_Δ2), we note significant protection of the H3 α3 helix

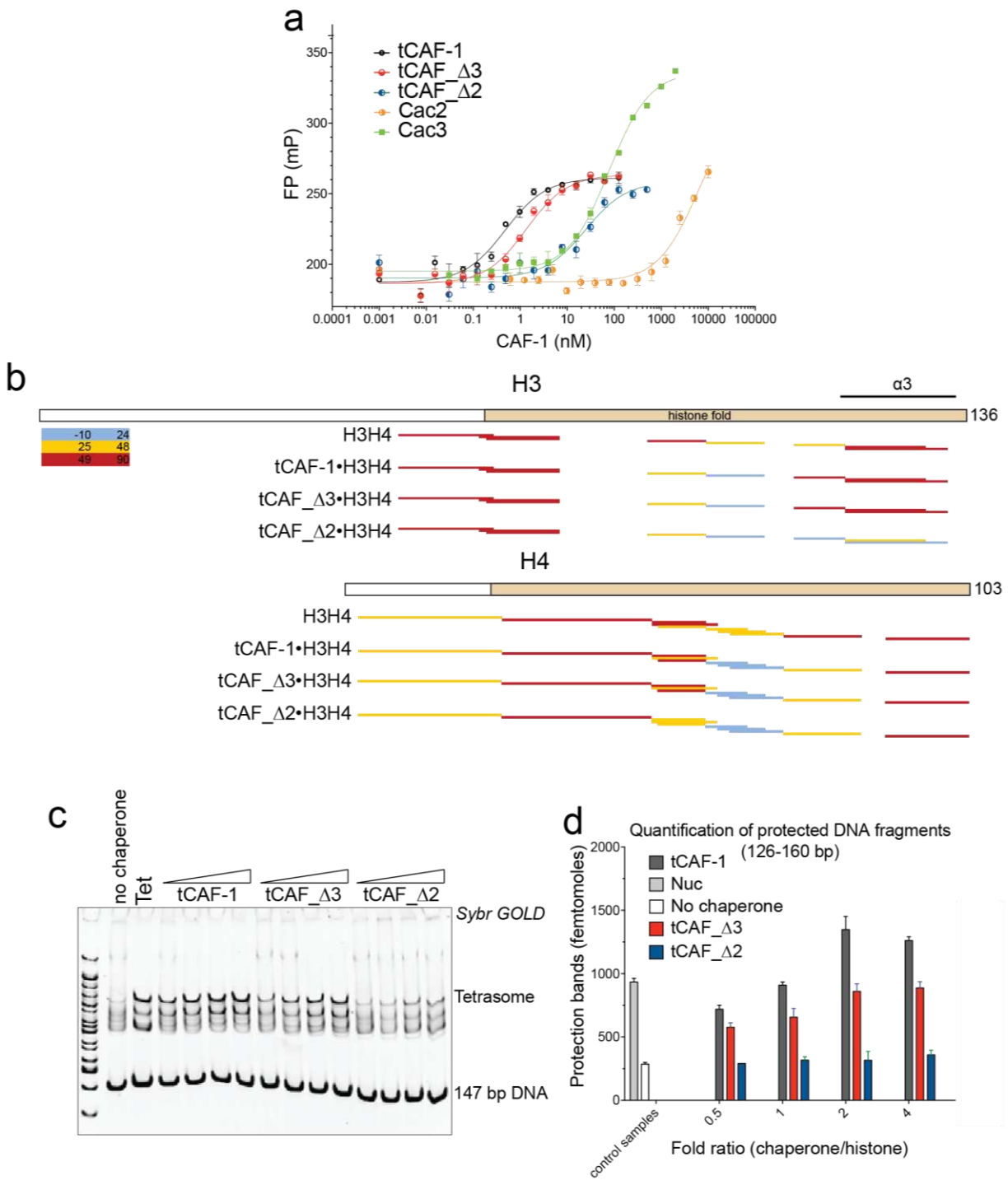


Figure 2.5 Cac2 is essential for proper histone binding in CAF-1

a) Affinity measurements (fluorescence polarization) of CAF-1 subunits and sub-complexes to H3-H4 labeled with Alexa488. Values are reported in Table 1. b) HX-MS heatmap of deuteron uptake for histone H3 and H4 peptide (histone alone or in complex with different CAF-1 substrates). The deuterium update percentage for each peptide was calculated and scored. The results for tCAF-1 are published in Mattiroli et al. Supplementary File 2 (seen https://www.nature.com/articles/srep46274#s1) contains all the HX uptake values for H3-H4

shown in this figure panel. c) Tetrasome assembly assay with 147bp 601 DNA. Products were resolved on a 6% native gel. $(H3\text{-}H4)_2$ tetramer concentration is 100 nM, chaperones are titrated with 0.5, 1, 2 and 4 fold over tetramer concentration, DNA is 100 nM. d) Quantification of the nucleosome bands (126-160 bp) from the NAQ assays. The nucleosome assembly reactions on 207 bp DNA were treated with MNase. The DNA was then purified and quantified from Bioanalyzer runs. Mean ±SEM is shown.

(H3 aa 110-126), the region responsible for $(H3\text{-}H4)_2$ tetramerization (Figure 2.5b). This region remains exposed when the histones are in complex with functional tCAF-1 and tCAF-1 lacking Cac3 (tCAF_Δ3) (Figure 2.5b). This suggests that in absence of Cac2, histones are bound in a different manner, supporting a pivotal role for Cac2 in organizing histone binding by CAF-1.

To understand whether this residual binding of tCAF_Δ2 to histones is sufficient to sustain nucleosome assembly by CAF-1, we performed a tetrasome assembly assay. Here, we observed no product formation in the absence of Cac2 (Figure 2.5c). This was confirmed when we measured nucleosome assembly activity of the tCAF-1 subcomplexes in a quantitative nucleosome assembly (NAQ) assay (Figure 2.5c). In this assay, we quantify the amount of assembled nucleosomes by treating assembly reactions with Micrococcal Nuclease (MNase), followed by quantification of length and amount of the purified DNA on a Bioanalyzer. These data demonstrate that Cac2 is indispensable for proper histone deposition onto DNA.

Importantly, although Cac3 has no significant effect on histone binding, its depletion has a minor effect on tetrasome and nucleosome formation (Figure 2.5c-d), suggesting that this subunit may contribute to the deposition process, possibly indirectly by organizing the Cac1 central region.

2.2.4 CAF-1 binds H3-H4 through a composite interface

Because the acidic region on Cac1 is located in close proximity to the Cac2 binding site mapped by HX-MS (Figure 2.3b), and thought to be involved in H3-H4 binding (Kaufman et al., 1995; Liu et al., 2016), we wanted to test if it formed a composite binding site for histones together with Cac2. We first asked whether the acidic region on Cac1, together with Cac1-bound Cac2, constitutes the minimal unit for high affinity H3-H4 binding. Indeed, a complex containing Cac2 and a Cac1 fragment encompassing only the acidic and the Cac2 binding region (Cac1_HB, Figure 2.6a) binds H3-H4 with a Kd of ~2 nM, which is within the same range as the affinities measured for CAF-1. We conclude that this complex represents a minimal histone binding module (HBM, Figure 2.6b and Table 2.1), sufficient for high affinity histone binding. When we

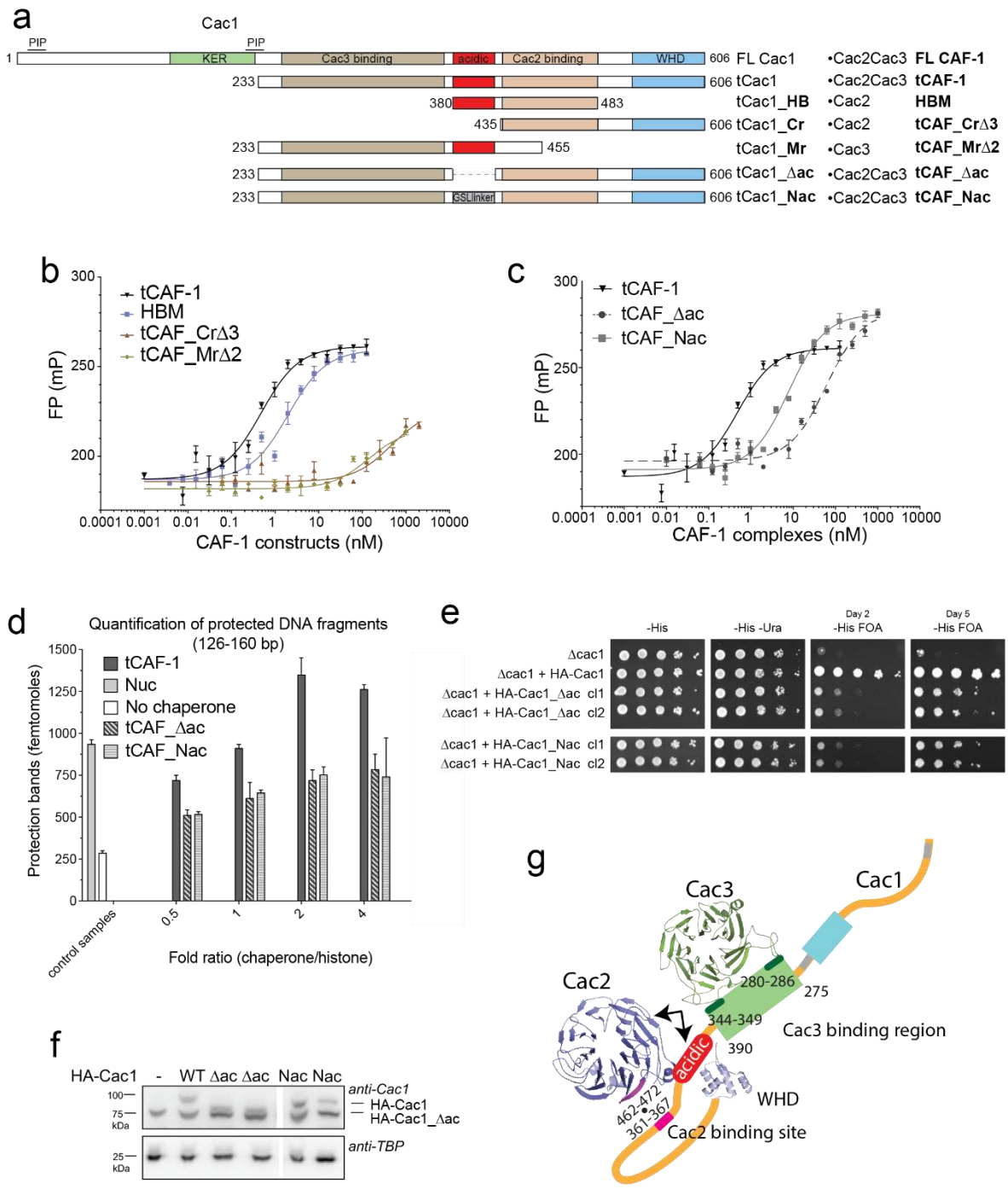


Figure 2.6 CAF1 binds histones H3-H4 through a composite interface

a) Schematic overview of the constructs used to validate histone binding and deposition mechanism. Summary of the binding affinity and the activities measured for each complex. PIP stands for PCNA-interacting peptide. WHD: winged-helix domain. KER : KER-rich (highly charged, pI = 9.89) domain. PIP: PCNA interaction peptide. HBM: histone binding module. Cr: C-terminal region of Cac1. Mr: middle region of Cac1. ∆ac: tCac1 with deleted acidic region. Nac: tCac1 with neutralized acidic region. b) Affinity measurements (fluorescence polarization) of CAF-1 subunits and sub-complexes described in figure 2.6a, to H3-H4 labeled with Alexa488. Values are reported in Table 2.1. c) Affinity measurements (fluorescence polarization) of tCAF-1, tCAF_∆ac and tCAF_Nac, to H3-H4 labeled with Alexa488. Values are reported in Table 2.1. d) Quantification of the nucleosome bands (126-160 bp) from the NAQ assays of tCAF-1, tCAF_∆ac and tCAF_Nac. The nucleosome assembly reactions on 207 bp DNA were treated with MNase. The DNA was then purified and quantified from Bioanalyzer runs. Mean ±SEM is shown of at least two independent experiments. e) Silencing assay performed with yeast strains expressing the Cac1 mutations at the acidic region. Samples were spotted at 0-10^1-10^2-10^3-10^4 dilutions from a OD600=1 stock. f) Immunoblot analysis to confirm expression of Cac1_∆ac and Cac1_Nac proteins in yeast cells used for the experiments shown in Figure 4e. The full-length blots are shown in panel b. g) A yeast CAF-1 model showing the interfaces between the 3 subunits. Histone binding occurs through a composite histone binding interface on CAF-1, including the acidic region of Cac1 and Cac2 (arrows). Data from SEC-SAXS and HX-MS were used to build this structural model. The Cac2 and Cac3 homology models were generated using Phyre2.

use a Cac1 construct spanning the C-terminal region (Cr) but lacking the acidic region (tCAF_CrΔ3, Figure 2.6a) we lose H3-H4 binding (Figure 2.6b and Table 2.1). Similarly, H3-H4 binding is lost in a Cac1 construct containing the middle region (Mr), including the acidic patch but lacking the Cac2 binding site (tCAF_MrΔ2, Figure 2.6a-b and Table 2.1). Finally, we prepared trimeric tCAF-1 complexes where we either deleted the acidic region in Cac1 (tCAF_Δac) or replaced it with a neutral Gly-Ser-Leu peptide of the same length (tCAF_Nac) (Figure 2.6a). tCAF_Δac had a markedly reduced affinity for H3-H4 (Kd = 60 nM, Figure 2.6c and Table 2.1), while tCAF_Nac had a less pronounced effect (Kd = 8 nM, Figure 2.6c and Table 2.1). These data demonstrate that the charges in the Cac1 acidic region are important for high affinity histone binding, yet histone binding affinity could be partially rescued by replacing this region with a neutral spacer. These data indicate that the acidic region not only functions through its charged nature, but that it also may exert a structural role in shaping the relative orientation of the Cac2 and the Cac3 binding regions on Cac1.

Notably, a minor effect in nucleosome assembly is seen with these mutants in the NAQ assay (Figure 2.6d), indicating that the reduced binding affinity (~10 fold) does not completely preclude CAF-1 function *in vitro*. Nonetheless, when testing these mutations for their transcriptional silencing effect in cells, we observed delayed growth on FOA plates, consistent with a defect in proper CAF-1 function (Figure 2.6e-f).

Together these data indicate that the acidic region contributes to the histone binding interface together with Cac1-bound Cac2, and its integrity is important for CAF-1 function in cells. Notably, the more severe effects observed in the NAQ assay in the absence of Cac2 (tCAF_Δ2, Figure 2.5c) suggest that in the context of the CAF-1 complex, Cac2 is the cornerstone of the CAF-1•histones binding interface.

2.3 Discussion

In this study, we show that CAF-1 has an elongated shape in solution, and that the extended large subunit Cac1 bridges Cac2 and Cac3, which themselves do not interact with each other. By using HX-MS followed by site directed mutagenesis, we identified peptides that form the interfaces between the three subunits of CAF-1 (Figure 2.6g). Cac3 binds to the Cac1 middle domain N-terminally to the acidic region, through two main points of contact on Cac1, and Cac2 interacts through a single hydrophobic interaction with the Cac1 C-terminal region located between the acidic region and the winged helix domain (WHD, Figure 2.6g). Using yeast genetics, we show that these interfaces are relevant *in vivo*. Together, these data further refine recently published cross-linking data (Kim et al., 2016; Liu et al., 2016). Furthermore, the mutants described here can be used *in vivo* to assess CAF-1 subunit contributions, without the need to deplete these proteins, hence minimizing secondary effects.

Previous studies using Cac3 homologues (human and Drosophila) in isolation suggested that the smallest CAF-1 subunit may be responsible for histone binding by CAF-1(Nowak et al., 2011; Schmitges et al., 2011; Song et al., 2008; Zhang et al., 2013). Using quantitative assays, we show that Cac3 has only minor contributions to H3-H4 binding within CAF-1. Apparently, in the context of this complex, the histone binding capacity of this subunit is not utilized as a primary interface. Nonetheless, our data suggest that Cac3 helps orchestrate the complex series of events that are required for the nucleosome assembly process, potentially by organizing the Cac1 central region. In fact, upon removal of Cac3, HX changes were observed throughout a ~115 residues region of Cac1, accounting for 19% of FL Cac1 residues and 30% of tCac1. It is possible that in absence of Cac3 this region becomes disordered, thereby interfering with the molecular mechanism of CAF-1 mediated chromatin assembly.

We show that the middle subunit Cac2 is required for productive histone binding, but only when in complex with Cac1, as it does not bind histones in isolation. This suggests that the Cac1-

Cac2 interaction shapes the histone binding interface on CAF-1, which also includes the Cac1 acidic region, located in proximity to the Cac2 binding site. Our analysis demonstrates that in absence of Cac2 (tCAF_Δ2), histones are bound in a non-productive form, as they exhibit a different HX protection pattern, and their assembly into tetrasomes is completely inhibited. It remains yet to be resolved whether Cac2 directly interacts with H3-H4 within CAF-1, or whether it plays a structural role that indirectly affects histone binding. Notably, the location of the Cac2-Cac1 interface places Cac2 at the nexus of the CAF-1 region responsible for histone deposition. Data from our lab (Mattiroli et al, related manuscript) shows that the Cac1 C-terminal WHD, which is a DNA binding motif(Zhang et al., 2016a), coordinates nucleosome assembly, and its action is regulated by an intramolecular inhibitory interaction with the H3-H4 histone binding site, in particular the Cac1 acidic region. As the Cac2 binding site on Cac1 is located between these two Cac1 domains, it is plausible that Cac2 further controls the structural re-arrangements occurring on CAF-1 during the nucleosome assembly process. These data, together with the contribution of the middle subunit to the interaction with the histone chaperone Asf1(Malay et al., 2008; Mello et al., 2002; Tang et al., 2006; Tyler et al., 2001) places Cac2 at the heart of the histone binding activity of CAF-1 during chromatin assembly in DNA replication.

2.4 Materials and methods

2.4.1 Cloning and reagents

cDNA for the three yeast CAF-1 subunits were a gift from Paul Kaufman. These were cloned into the MultiBac vector for expression in insect cells. Cac1 was cloned into pACEBac1, Cac3 in pIDC and Cac2 in pIDS and these were recombined by Cre-Lox as described(Bieniossek et al., 2008). A His-tag was inserted at the C-terminal end of Cac2 for purification purposes. All complexes are prepared with a short C-terminal deletion of Cac2 (1-449) to remove a region predicted to be highly disordered. Bacmids for expression in SF21 cells were prepared as previously described(Bieniossek et al., 2008). Mutations were introduced using Turbo Pfu

polymerase (Roche) in a standard mutagenesis protocol. N-terminal sequencing was performed at the Protein Facility of Iowa State University. The result from the Edman degradation identify the Cac1 fragment starting with sequence FFKKLS. This corresponds to Cac1 residues 233-238. The structural models of Cac2 and Cac3 were generated using Phyre2 in intensive mode(Kelley et al., 2015).

2.4.2 Protein purification

Histones were purified from E.coli cells as previously described(Dyer et al., 2004), and stored in 2 M NaCl at -80°C. Labeling of histone proteins was performed as previously described(Muthurajan et al., 2016). We labeled H4 with AttoN-647 on T71C or E63C, and H2B with Alexa488 on T112C prior refolding with the histone partner. yCAF-1 was expressed in SF21 cells and purified using a HisTrap column (GE) in buffer containing 50 mM TRIS 8.0, 600 mM NaCl, 5% glycerol, 10 mM imidazole, 5 mM BME (beta-mercaptoethanol), in presence of COMPLETE EDTA-free, DNase I, 3 mM CaCl2 and 3mM MgCl2. The complex was then loaded on a MonoQ column in buffer A (50 mM TRIS 8.0, 200 mM NaCl, 1 mM EDTA, 1 mM TCEP) and eluted with buffer B containing 1 M NaCl. The protein was then injected into a size exclusion column (Superdex 200) in buffer 30 mM TRIS pH 7.5, 300 mM NaCl, 1 mM EDTA, 1 mM TCEP. For HX-MS studies, buffer containing 50 mM KPO4, 150 mM NaCl, 5 mM DTT (pH 7.4) was used for the gel filtration step. Proteins were concentrated to 1-20 mg/ml and stored at -80°C in their gel-filtration buffer. Mutants were purified as wild-type proteins. All mutant complexes were well-behaved and did not exhibit abnormalities during the purification procedure. The tCAF-1 complexes contain a deletion or mutation of the acidic region in Cac1 were purified over a MonoS, instead of the MonoQ column with buffer A and B at pH 6.8.

2.4.3 Tetrasome assembly assays

The assays were carried out in buffer containing 25 mM TRIS pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.02% Tween-20, 0.5 mM TCEP. CAF-1 was first diluted at different concentrations;

normally a chaperone-histone ratio between 0.5 to 4 fold was used. Histones H3-H4 (100 nM tetramer concentration) were added, and the chaperone-histone mix was incubated at room temperature for 10 minutes. DNA was then added at 100 nM concentration. The reactions were incubated for 10-30 minutes (no differences were observed when incubating for longer time). Glycerol was added to a final concentration of 10% v/v prior loading of the samples on a 6% PAGE gel, pre-run in 0.2x TBE buffer at 4°C. The gels were run for 70 minutes at 150 V at 4°C. Gels were stained with SybrGOLD for 10 minutes and imaged on a Typhoon FLA 9500 at 488 nm.

2.4.4 NAQ assay (Nucleosome assembly and quantification)

The assembly assay was carried out as described above containing 200 nM of 207 bp DNA, 200 nM $(H3\text{-}H4)_2$, 400 nM H2A-H2B and titration of CAF-1 (100-200-400-800 nM). After the assembly reaction, the samples were diluted to a DNA concentration of 50 nM in 100 µl digestion reactions. 25 U of MNase enzyme was added in a final buffer containing 50 mM TRIS pH 7.9, 5 mM CaCl2. After incubation at 37°C for 10 minutes, the reactions were quenched with 10 µl of 500 mM EDTA, pH 8. The DNA was then purified using a modified protocol of the MinElute kit from QIAGEN. 550 µl of PB buffer and 10 µl of 3M sodium acetate were added to each sample and they were incubated at room temperature for 10 minutes. At this point, 50 ng of DNA loading control (or reference band, a 621 bp DNA fragment) was added to each tube. The samples were applied to the MinElute spin column and washed as prescribed by QIAGEN. The DNA was eluted with 10 µl of water. 1 µL of the eluate was used to load a DNA 1000 chip on the Bioanalyzer machine (Agilent), and 2.5 µl were loaded on a 10% PAGE gel. The gel was run for 45 minutes at 200 V in 0.5x TBE buffer at room temperature. Gels were stained with SybrGOLD for DNA and imaged on a Typhoon FLA 9500 (GE). The Bioanalyzer data was analyzed using the Agilent Expert 2100 software. The reference band was corrected for the proper size (621 bp) and the calculated molarity values were used to normalize all other bands

present in the lane. The normalized values were used in the quantification and comparison. The signal threshold was set at 20 RFU. Nucleosome signal was calculated from bands ranging between 126-160 bp in length, based on the digestion of salt-reconstituted nucleosomes.

2.4.5 Fluorescence Polarization experiments

Fluorescence Polarization assays were carried out in 25 mM TRIS pH 7.5, 300 mM NaCl, 5% glycerol, 1 mM EDTA, 0.01% NP-40, 0.01% CHAPS, 1 mM DTT (added fresh). Binding reactions were prepared by mixing 5 nM of Alexa488-labeled H3-H4 dimer and increasing amounts of CAF-1. Binding data were measured using a BioTek Synergy H2 plate reader. The data was analyzed and plotted using Microsoft Excel and GraphPad Prism.

2.4.6 Size Exclusion Chromatography in line with Multi-Angle Light Scattering (SEC-MALS)

A Superdex 200 10/300 GL (GE) was mounted in line with a DAWN HELEOS II light scattering and a Optilab rEX refractive index detectors (Wyatt). The runs were performed in 20 mM TRIS pH 7.5, 300 mM NaCl, 1 mM EDTA, 1 mM TCEP at room temperature. 100 µL of protein sample at ~3 µM were injected at 0.3 ml/min, after being spun down at 14000 g for 10 minutes. Data analysis was done using the ASTRA software (Wyatt) and GraphPad Prism was used to prepare figures.

2.4.7 Size Exclusion Chromatography in line with Small Angle X-ray Scattering (SEC-SAXS)

Superdex 200 (GE) 10/300 GL was mounted in line with the SAXS beamline at APS, Chicago. The runs were performed in 20 mM TRIS pH 8.0, 300 mM NaCl, 1mM DTT at room temperature (25 °C). 500 uL of the FL CAF-1 complex was injected into the column at 1 mg/ml. Data was collected at APS, Beamline 18-ID-D with 1 sec exposures every 5 seconds, at 1.03 Å (12Kev). The camera length was ~3.5m, the q-range we accessed was 0.0047 Å-1 to ~0.25 Å-1 and the detector used was a Mar165 CCD. Data reduction and buffer subtraction were done using the

beamline-specific pipeline. The ATSAS software package was used for analysis(Petoukhov et al., 2012). After data analysis with primus, 10 independent ab initio models were generated with DAMMIF, with NDS values ranging between 0.483 and 0.695. These were then averaged with DAMAVER and DAMFILT generated the final model. The data shown here was generated based on the average curve between the five fractions coinciding to the highest absorbance/scattering during elution from the column. The results are very similar when each fraction was analyzed individually. GraphPad Prism and pdb2vol/VMD were used to prepare figures.

2.4.8 Hydrogen Deuterium Exchange coupled to Mass Spectrometry (HX-MS)

HX was performed on stock samples of the CAF-1 complexes and/or histones at 4 µM in HX buffer (50 KPO4, 150 mM NaCl, 5 mM DTT pH 7.4). The CAF-1 complexes were gel filtered into HX buffer, and histones were dialyzed overnight in the HX buffer to ensure that the final samples would not contain any additional buffer components that may result in buffer variability between proteins. HX was initiated by mixing 5 µl of the 4 µM stocks with 45 µl of deuterated HX buffer (prepared by dissolving lyophilized HX buffer in 99.9% D2O) to result in an approximate final 90% D2O concentration. Exchange occurred for either 30 seconds, 1, 10, 30, or 60 minutes at 10ºC. Exchange was quenched by adding 50 µl of ice cold quench buffer (25 mM succinic acid, 25 mM citric acid at pH 2.2), that brought the reaction mixture to pH 2.4. Pre-quenched control reactions were prepared by adding quench buffer prior to D2O buffer. The samples were immediately injected into a temperature controlled (0 ºC) Waters HDX Manager for online proteolysis at 12ºC using an immobilized pepsin column (Life Technologies), followed immediately by peptide trapping and an online 3 min desalting step at 0 ºC using a Waters BEH UPLC C18 trap column for 3 min, all with 100% solvent A (0.1% formic acid in water) flow at 100 µL/min. Peptides were then separated at 0 ºC using a Waters 100 mm BEH C18 analytical UPLC column and a linear 8% to 40% solvent B (0.1% formic acid in acetonitrile) gradient over

6 min, followed by a 1 min 40% B hold and subsequent ramp to 85% solvent B in 0.5 min using a Waters nanoAcquity UPLC and 40 µL/min flow rate. The UPLC was coupled directly to a Waters Synapt G2 HDMS q-TOF mass spectrometer operating in positive, MSe data acquisition mode. Samples were run and analyzed in a random order. Non-deuterated, prequenched (indicated as 0.01 sec samples in the uptake plots), 1 and 60 minutes samples were taken in triplicates.

PLGS 3.0 (Waters) was used to create an identified peptide list from non-deuterated datasets and DynamX 3.0 (Waters) performed the search for deuterated peptide ion assignments. All isotope assignments for each peptide in each charge state were manually verified. The weighted average mass of each peptide determined by DynamX was then used to calculate deuteron uptake which was converted to % deuteration based on the number of maximum exchangeable amide protons. Data were corrected for artefactual in-exchange using the quenched experiment as previously reported(Sours and Ahn, 2010). No corrections for back-exchange were conducted due to the comparison of relative uptake amounts between bound and unbound states, which would remain unaffected by the back-exchange correction. Graph bars and uptake plots were prepared using Microsoft Excel and GraphPad prism. The uptake data of all the peptides analyzed in this study are shown in Supplementary Files 1 and 2.

2.4.9 Yeast heterochromatin maintenance *in vivo* assay

The endogenous CAC1 locus was cloned into a pRS313 vector (the fragment contained 659 bp upstream and 731 bp downstream the Cac1 ORF). An HA-tag was introduced in the 5' end of the ORF. This construct was used to transform the PKY106 strain obtained from PD Kaufman(Kaufman et al., 1997). Mutants were generated using site-directed mutagenesis and were treated as the WT sample. The empty pRS313 vector was used as control. Transformed PKY106 strains were grown in synthetic media lacking histidine (-His). Clones were amplified overnight and then diluted in the morning and grown to an OD600 = 0.7-0.8. After washing the

cells with water and then resuspending them to an OD600 = 1, four 10-fold dilutions from the initial stock were prepared. The undiluted sample and these dilutions were spotted on plates containing -His or -His-Ura media as controls, and -His supplemented with 1 mg/ml 5-Fluoroorotic Acid (FOA) for growth selection. Plates were left at 30°C for 2 days and then left at room temperature for up to the 7th day. To validate HA-Cac1 expression, 7 ml of cultures were harvested at OD600 = 0.7-0.8 and washed in water. The pellet was boiled for 3 minutes. 50 µl of PBS buffer containing 1 mM TCEP and COMPLETE EDTA-free protease inhibitors was added to the pellet. Cells were lysed using glass beads and the lysate was then spun down. The supernatant was loaded on a SDS PAGE gel and transfer to a PVDF membrane. The blot was probed with anti-Cac1 antibodies (a gift from Zhiguo Zhang).

CHAPTER 3: DNA-MEDIATED ASSOCIATION OF TWO HISTONE-BOUND CAF-1 COMPLEXES DRIVES TETRASOME ASSEMBLY IN THE WAKE OF DNA REPLICATION[2]

Overview

Nucleosome assembly in the wake of DNA replication is a key process that regulates cell identity and survival. Chromatin assembly factor 1 (CAF-1) is a H3-H4 histone chaperone that associates with the replisome and orchestrates chromatin assembly following DNA synthesis. Little is known about the mechanism and structure of this key complex. Here we investigate the CAF-1•H3-H4 binding mode and the mechanism of CAF-1 mediated nucleosome assembly. We show that CAF-1 binding to a H3-H4 dimer activates the Cac1 winged helix domain (WHD) interaction with DNA. This drives the formation of a transient CAF-1•histone•DNA intermediate containing two CAF-1 complexes, each associated with one H3-H4 dimer. Here, the $(H3\text{-}H4)_2$ tetramer is formed and deposited onto DNA. Our work sheds light on the molecular mechanism for histone deposition by CAF-1, a reaction that has remained elusive for other histone

[2] Declaration of contributions:

This chapter was published (Mattiroli et al., 2017b) as a collaboration between the following authors: Francesca Mattiroli*, Yajie Gu*, Tejas Yadav, Jeremy L. Balsbaugh, Michael R. Harris, Eileen S. Findlay, Yang Liu, Catherine A. Radebaugh, Laurie A. Stargell, Natalie G. Ahn, Iestyn Whitehouse, Karolin Luger. I chose to include this full manuscript into this chapter because the presented data has been peer-reviewed and the conclusions out of this manuscript partially fulfil the goal of my project. I am responsible for the cloning, expression and purification of full length CAF-1, tCAF-1, tCAF_Δ3, Cac2, HBM, tCAF_ MrΔ2, tCAF_CrΔ3, tCAF-1_WHD**, tCAF-1_ΔWHDa, and tCAF-1_ΔWHDb used in this paper. The HX-MS was performed in full collaboration with Francesca Mattiroli. I performed studies shown in Figures 3.1 a, b, c, d; 3.2 a, b, c, d, e; 3.3 a, b, d, e, f; 3.4 b; 3.5 b; 3.6 b, c; 3.7 a (partial), c, d; 3.8 b, c; 3.9 e; 3.10 a; 3.11 d, e; 3.13 a, b, c; 3.14 a. Dr. Mattiroli and I were equally responsible for interpreting the data.

chaperones, and it advances our understanding of how nucleosomes and their epigenetic information are maintained through DNA replication.

3.1 Introduction

Dynamic assembly and disassembly of nucleosomes regulates accessibility to the genome during the processes of DNA transcription, replication and repair. DNA replication constitutes a particularly challenging context, as nucleosomes and the epigenetic information they encode need to be reestablished and duplicated onto daughter strands (Alabert and Groth, 2012). This process involves a number of histone chaperones that operate through a network of sequential interactions (Groth et al., 2007a; Huang et al., 2015b; Jasencakova et al., 2010; Richet et al., 2015; Saredi et al., 2016; Smith and Stillman, 1989; Tyler et al., 1999). Histone chaperones bind histones (Elsasser and D'Arcy, 2013), and are responsible for the maintenance of nucleosome density and the faithful inheritance of the epigenetic information.

While knowledge of how histone chaperones bind histones is steadily increasing (Huang et al., 2015b; Mattiroli et al., 2015; Richet et al., 2015; Ricketts et al., 2015; Saredi et al., 2016), the mechanism and structural transitions required for histone deposition onto DNA remain unknown. This is a non-trivial process in light of the complex architecture of the histone octamer in the nucleosome, requiring the ordered deposition of histone pairs. This is particularly relevant for the first step of nucleosome assembly, the deposition of the $(H3\text{-}H4)_2$ tetramer (tetrasome formation).

Parental and newly-synthesized H3-H4 transition between a dimeric and a tetrameric state, while replication-coupled histone chaperones distribute them onto the daughter strands during DNA replication (Campos et al., 2010; Clement and Almouzni, 2015; English et al., 2006; Huang et al., 2015b; Richet et al., 2015). Current evidence favors a conservative model for H3-H4 inheritance, where parental $(H3\text{-}H4)_2$ tetramers are not split and distributed onto the daughter

strands but rather deposited as one unit (Xu et al., 2010), but alternative models have also been proposed (Tagami et al., 2004).

CAF-1 is the key nucleosome assembly factor associated with DNA replication (Smith and Stillman, 1989). CAF-1 directly interacts with the replisome via the processivity factor PCNA (Krawitz et al., 2002; Moggs et al., 2000; Rolef Ben-Shahar et al., 2009; Shibahara and Stillman, 1999; Zhang et al., 2000) and bridges interactions with epigenetic factors (Loyola et al., 2009; Murzina et al., 1999; Quivy et al., 2008). The unique role of CAF-1 in integrating chromatin assembly with DNA synthesis and epigenetic signaling makes it indispensable for the maintenance of cell identity and for life in multicellular organisms (Barbieri et al., 2014; Cheloufi et al., 2015; Houlard et al., 2006; Ishiuchi et al., 2015; Nakano et al., 2011; Song et al., 2007). In yeast, CAF-1 deletion is viable but results in aberrant transcriptional silencing programs and sensitivity to DNA damage (Kaufman et al., 1998).

CAF-1 is a histone H3-H4 chaperone, composed of three distinct subunits, all of which are conserved from yeast (named Cac1, Cac2 and Cac3) to humans (Almouzni and Mechali, 1988; Kaufman et al., 1995; Kaufman et al., 1997; Smith and Stillman, 1989; Tyler et al., 1999; Verreault et al., 1996). Previous biochemical studies have suggested that one CAF-1 complex can bind more than one H3-H4 dimer (Liu et al., 2012), and other studies have proposed models where CAF-1 dimerization may be important for its function *in vivo* (Nakano et al., 2011; Quivy et al., 2001). To date, the mechanism by which CAF-1, and indeed any histone chaperone, assembles the $(H3\text{-}H4)_2$ tetramer onto DNA is unknown. Understanding how CAF-1 deposits H3-H4 is essential for our understanding of the mechanisms that govern the inheritance of epigenetic modifications and epigenome maintenance.

Here, we describe the mechanism by which CAF-1-mediates deposition of the $(H3\text{-}H4)_2$ tetramer onto DNA. A coordinated sequence of events is set in motion by H3-H4 binding to CAF-1, and promoted by CAF-1•DNA interaction. The mechanism culminates in the DNA-

mediated association of two CAF-1•H3-H4 complexes to form the $(H3\text{-}H4)_2$ tetramer, which is then transferred to DNA. These findings elucidate the histone deposition mechanism by this key histone chaperone complex, with significant implications on our understanding of chromatin propagation in DNA replication.

3.2 Results

3.2.1 tCAF-1 is sufficient for nucleosome assembly in vitro

Whether recombinant CAF-1 assembles nucleosomes *in vitro* in absence of the replication machinery or other nuclear proteins remains an open question (Gaillard et al., 1996; Kadyrova et al., 2013; Kaufman et al., 1995; Smith and Stillman, 1991b), which we set out to resolve in a purified *in vitro* system. We developed a quantitative nucleosome assembly assay (NAQ). Commonly used nucleosome assembly assays rely on native gel-based readouts, where the formation of the canonical nucleosome band is monitored over a range of histone chaperone concentrations. This readout is not suitable for proteins that bind DNA or the histone•DNA products, such as FL CAF-1 (Figure 3.1a, lanes 3-6, and Figure 3.2a). Therefore, we added a Micrococcal Nuclease (MNase) digestion step that allows us to monitor the DNA protection pattern induced by histone deposition. A further DNA purification step and addition of a DNA loading control allows accurate size determination and quantification of the recovered DNA fragments, hence the formation of nucleosomes.

We used the NAQ assay to study the *in vitro* nucleosome assembly activity of FL CAF-1 and of a truncated tCAF-1 complex lacking the first 233 amino acids in Cac1, but containing all the determinants for CAF-1 subunit assembly and histone binding (Mattiroli et al., related manuscript). The nucleosome assembly reactions on 207 bp DNA (Figure 3.1a) suggested efficient nucleosome formation by both FL and tCAF-1. Notably, tCAF-1 does not bind DNA or its products (Figure 3.1a, lanes 7-11), suggesting that the N-terminal portion of Cac1 mediates DNA binding observed with FL CAF-1. The assembly reactions were subjected to MNase

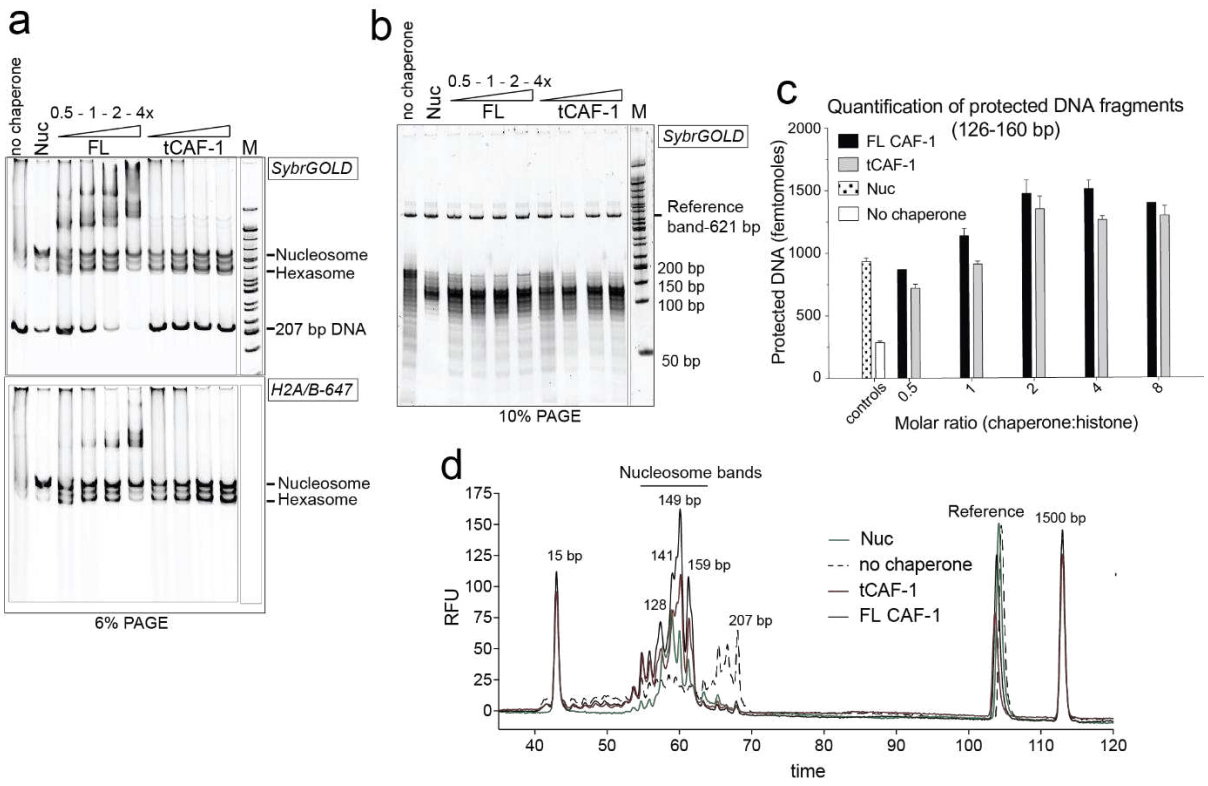


Figure 3.1 tCAF-1 is competent for nucleosome assembly in absence of other factors

a) Products from the nucleosome assembly assay on 207 bp DNA, with FL CAF-1 or tCAF-1. DNA is 200 nM, $(H3\text{-}H4)_2$ 200 nM and H2A-H2B-AttoN647 400 nM; 100-200-400-800 nM CAF-1 is titrated. Assemblies were analyzed by native PAGE. b) Products of MNase digestion performed on samples shown in panel a, after DNA purification. The 207 bp band in lane 1 results from nonproductive association of histones with DNA that renders it MNase-resistant. The disappearance of the 207 bp protection as CAF-1 is titrated demonstrates the ability of CAF-1 to relieve non-specific histone-DNA complexes, as observed for other chaperones. c) Quantification of protected nucleosomal DNA (126-160 bp) obtained from the samples shown in panel a and b. The reference DNA is used to normalize amounts in each lane. Mean ± SD is shown for at least three repeats. The data used for this panel is included in Figure 3.1- Source Data 1 (seen https://elifesciences.org/articles/22799/figures#SD4-data). d) Bioanalyzer electropherograms of protected DNA fragments from nucleosome assembly with 400 nM FL CAF-1 and tCAF-1 shown in b, with controls from salt-assembled nucleosomes (Nuc) and a no-chaperone sample.

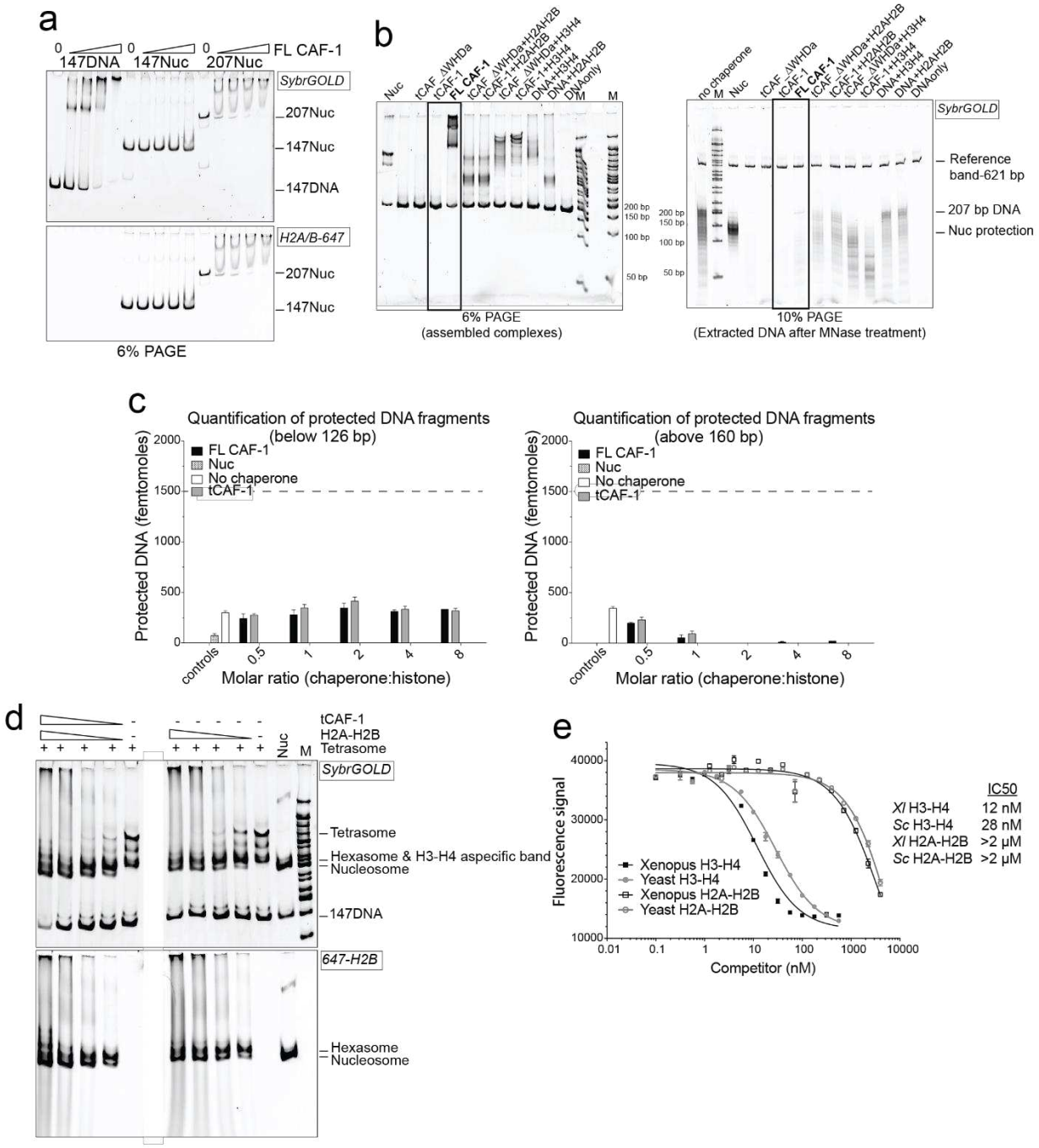


Figure 3.2 tCAF-1 is competent for nucleosome assembly

a) FL CAF-1 binds DNA that is not assembled into nucleosomes. DNA and salt-assembled nucleosomes (Nuc) are 50 nM; CAF-1 was titrated from 50-100-200-400 nM. b) DNA protection at 126-160 bp from the NAQ assay is specific for nucleosomes and hexasomes. Left panel: 6% PAGE of NAQ control reactions on 207 bp of DNA. Right panel: products of the MNase digestion performed on the same samples, after DNA purification. The black box highlights the control for the FL CAF-1•DNA and tCAF-1•DNA complexes, which show no significant protection after MNase treatment. These controls (lanes 5-6 on the left gel and 6-7 on the right

gel) demonstrate that DNA protection from MNase treatment is a result of nucleosome formation. The other controls confirm that deposition of either H3-H4 (lanes 10-12 on the left gel) or H2A-H2B (lanes 8-9 and 13 on the left gel) do not lead to significant DNA protection pattern. c) In addition to nucleosomes, no significant products are made by CAF-1 in the NAQ assay. NAQ assay quantification of protected bands below 126 bp (left panel) and above 160 bp (right panel), from the data shown in Figure 1c. The dashed line indicates the amount of nucleosome bands (i.e. 120-160 bp protection) assembled by CAF-1. The data use for this panel is included in Figure 3.1- Source Data 1(https://doi.org/10.7554/eLife.22799.003). Error bars show the SD from at least three replicates. d) H2A-H2B do not require histone chaperones to be incorporated into pre-assembled tetrasomes to form hexasome and nucleosomes. tCAF-1 has no additional effect on H2A-H2B association with the tetrasome. e) CAF-1 does not distinguish between yeast and Xenopus laevis histones, but has significantly lower affinity for H2A-H2B compared to H3-H4. Competition assays were performed with 10 nM of FL CAF-1•DMH3-H4 complex as a probe, where H4 was labeled with Alexa488 on T71C. Unlabeled Xenopus laevis or yeast histones were titrated to follow competition of the labeled H3-H4 dimer. Fluorescence quenching was monitored. Error bars show the S.E.M. from three independent measurements.

digestion, and the purified DNA was analyzed by native PAGE (Figure 3.1b) and quantified (Figure 3.1c-d). DNA fragments of about 125-160 bp accumulated with both CAF-1 constructs, similar to what was obtained with salt-assembled nucleosomes (Nuc, Figure 3.1b-d). To validate that these DNA fragments are representative of nucleosomes and not of other DNA•protein complexes, we performed a number of control reactions, including analysis of the CAF-1•DNA complexes, none of which result in significant DNA protection under these conditions (Figure 3.2b, box).

These experiments demonstrate that tCAF-1 and FL CAF-1 are both active and efficient nucleosome assembly factors in absence of other nuclear proteins. Our results also indicate that the N-terminal portion of Cac1, which *in vivo* is associated with PCNA, is not required for the nucleosome assembly activity, and that tCAF-1 contains all the requisite components for histone deposition. Nucleosome assembly activity increases in a CAF-1 dose dependent manner, and reaches its maximum at a two-fold excess of CAF-1 complex per $(H3\text{-}H4)_2$ tetramer (Figure 3.1c). No protected bands were detected above or below the nucleosomal DNA fragments (Figure 3.2c), confirming that the primary product of CAF-1 are nucleosomes and hexasomes. Because H2A-H2B can spontaneously associate with tetrasomes *in vitro* (Figure 3.2d), and because CAF-1 itself has significantly lower affinity for H2A-H2B compared to H3-H4 (Figure 3.2e), it appears that the primary role of CAF-1 is to promote the formation of an ordered (H3-H4)2•DNA complex, the tetrasome, as noted previously (Smith and Stillman, 1991b).

3.2.2 CAF-1 has one binding site for a H3-H4 dimer

To understand the mechanism of CAF-1 mediated nucleosome assembly, we first investigated how it binds histones. On CAF-1, the H3-H4 binding site is formed by the acidic region of Cac1 (Liu et al., 2016), in conjunction with Cac1-bound Cac2 (Mattiroli et al, related manuscript). Published data suggest that CAF-1 facilitates the formation of a $(H3\text{-}H4)_2$ tetramer (Liu et al., 2012; Liu et al., 2016). H3-H4 exists in an equilibrium between dimeric and tetrameric states in

absence of DNA or a histone chaperone. The estimated dissociation constant for tetramerization of the H3-H4 dimer is considerably weaker than the affinity of H3-H4 for DNA or for the histone chaperones (Donham et al., 2011). We therefore tested whether CAF-1 binds H3-H4 in its dimeric or tetrameric form. We used sedimentation velocity analytical ultracentrifugation (SV-AUC) to characterize the complexes formed between CAF-1 and histone H3-H4 combined at different molar ratios (1:1 or 1:2 CAF-1 to H3-H4 dimer), comparing wild type H3-H4 (WTH3-H4) with a constitutively dimeric H3-H4 mutant (DMH3-H4) that binds CAF-1 with the same affinity as WTH3-H4 (Figure 3.3a-b). Adding a 1:1 ratio of WTH3-H4 or DMH3-H4 dimer to CAF-1 resulted in the same homogenous shifts in S20,w value, seen both with FL and tCAF-1 (Figure 3.3c and 3.4a). Adding two WTH3-H4 dimers per CAF-1 molecule results in further apparent increase in size, possibly representing binding of a WT$(H3\text{-}H4)_2$ to one CAF-1. On the contrary, with DMH3-H4, which is unable to form a tetramer, we observed no additional increase (Figure 3.3c and 3.4a). These experiments were performed at 4 μM concentration where WTH3-H4 is expected to be primarily tetrameric, while DMH3-H4 remains a dimer. These data suggest that CAF-1 has a single binding site for H3-H4 and that this interaction may not affect tetramerization of $(H3\text{-}H4)_2$, as a shift to higher $S_{20,w}$ value is not observed with a dimeric DMH3-H4.

To further test this hypothesis, we used FRET-based Job plot experiments. Here, we used wild-type histones, constitutively dimeric DMH3-H4 and constitutively tetrameric XL$(H3\text{-}H4)_2$

prepared by cross-linking two H3-H4 dimers via a single cysteine residue on H3K115C (D'Arcy et al., 2013). This reaction yields a XL$(H3\text{-}H4)_2$ preparation where about 80% of the histones are tetramers (Figure 3.3d), in a conformation that is compatible with the (H3-H4)2 observed in the nucleosome structure. Notably, XL$(H3\text{-}H4)_2$ binds to FL or tCAF-1 with the same affinity as WTH3-H4 (Figure 3.3e). In stoichiometry experiments, performed under conditions where WTH3-H4 is primarily in its dimeric form, we observe one CAF-1 complex binding to one WT or

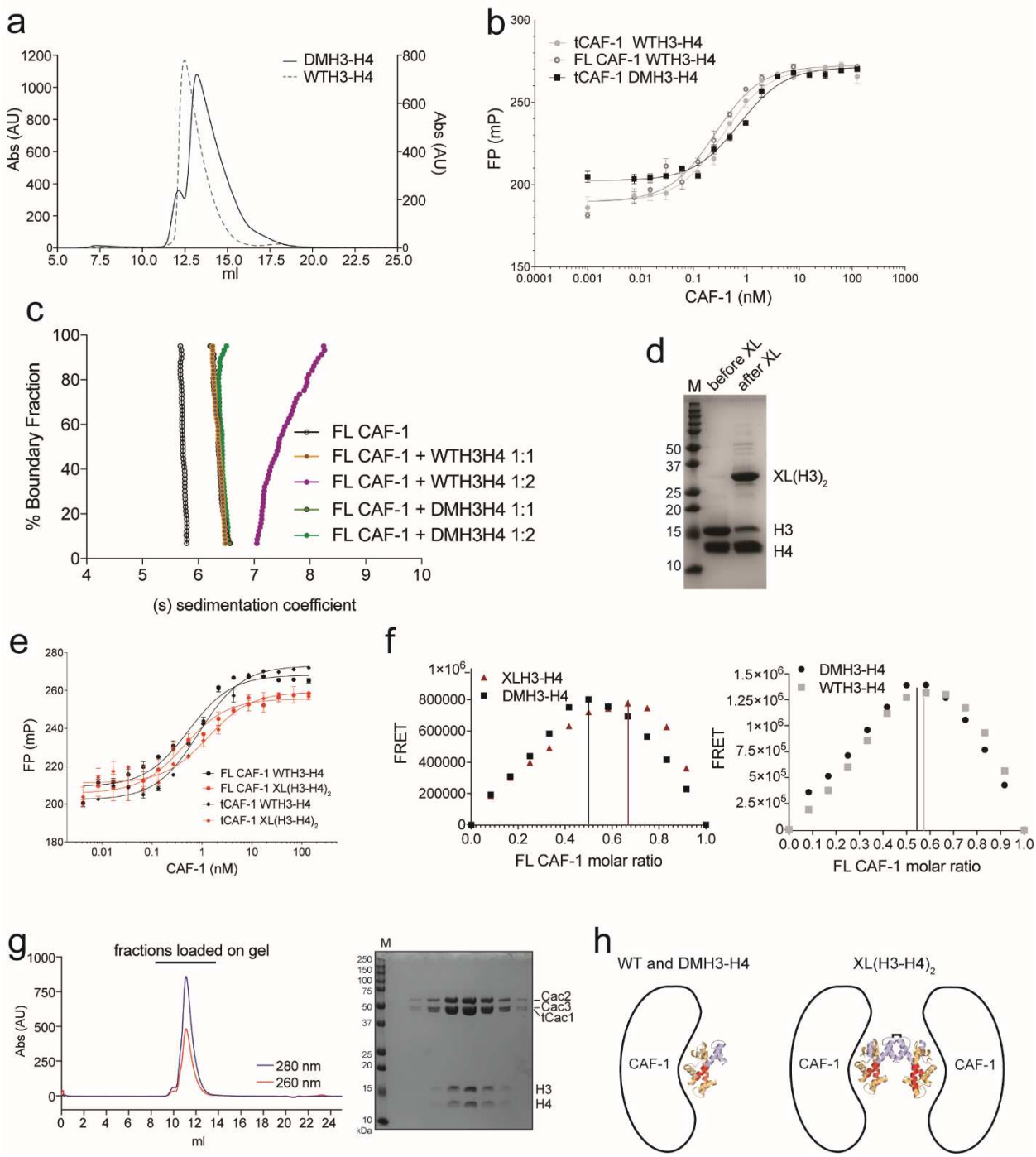
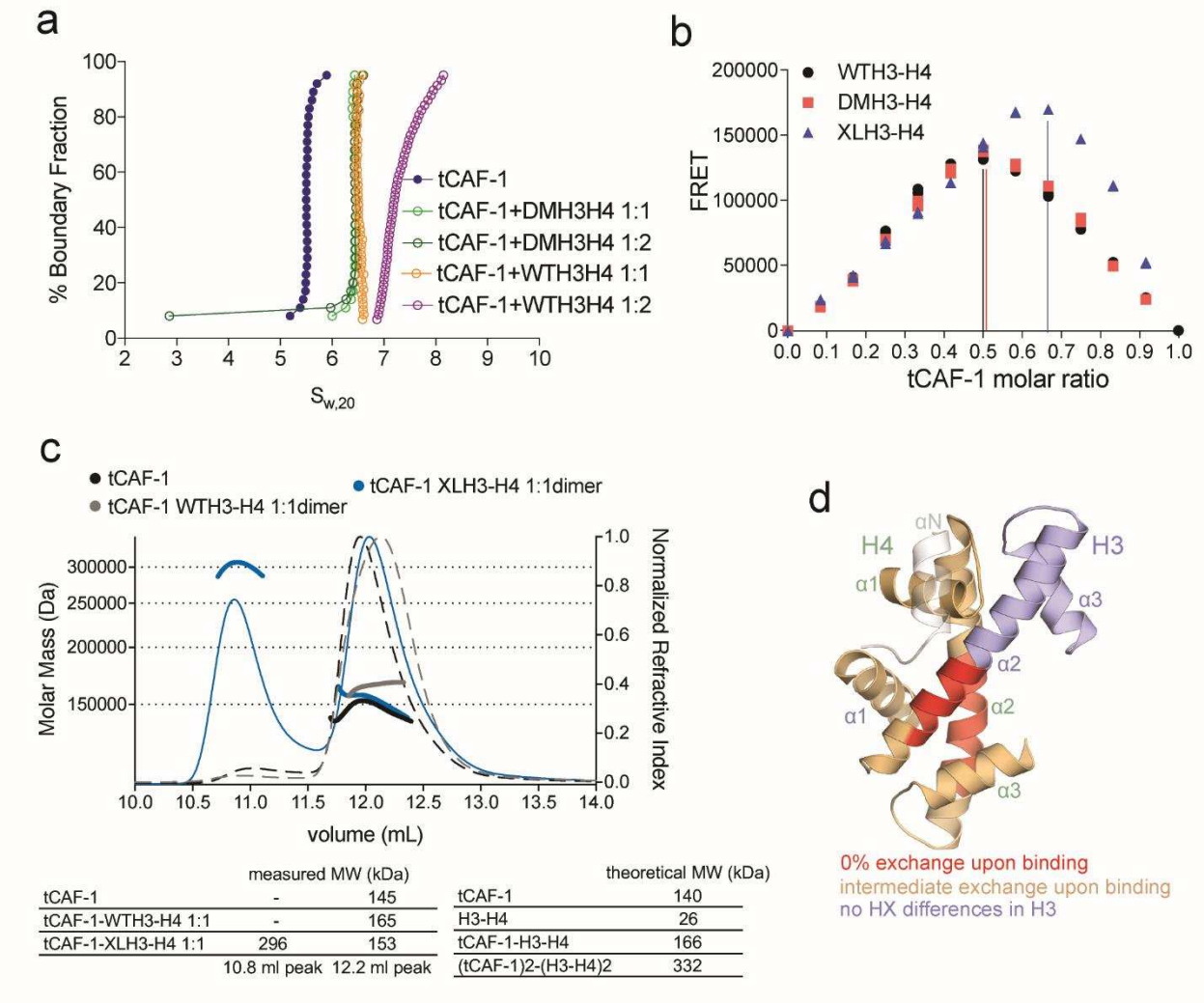


Figure 3.3 CAF-1 has a single binding site for a H3-H4 dimer

a) DMH3-H4 is primarily a dimer, even under conditions that favor the tetrameric state of WTH3-H4. Gel filtration of WTH3-H4 and DMH3-H4 in refolding buffer (2M NaCl). b) tCAF-1 binds equally well to WT or DMH3-H4, determined by fluorescence polarization (Kd ~ 0.5-0.7 nM). Error bars show the S.E.M. from three independent measurements. c) van Holde-Weischet analysis of SV-AUC experiments performed with FL CAF-1 and H3-H4 (wild type and DMH3-H4), combined in a 1:1 or 1:2 molar ratio of CAF-1 to H3-H4 dimer. An increased S-value upon addition of a second equivalent of H3-H4 dimer (but not with a second DM-H3H4) indicates that

a $(H3\text{-}H4)_2$ tetramer forms on CAF-1. d) SDS PAGE of histones before and after the chemical crosslinking to form XL(H3-H4)2. e) tCAF-1 binds equally well to WT or XL(H3-H4)2, as determined by fluorescence polarization (Kd ~ 0.5-0.9 nM). Error bars show the S.E.M. from three independent measurements. f) FRET-based Job plot experiment, using FL CAF-1 and either WT or DMH3-H4, shows a single binding event for both histone complexes (protein concentration was kept at 150 nM, where WT histones are dimeric). With XL(H3-H4)2, we observed a stoichiometry of two CAF-1 complexes per tetramer. Error bars show the SEM of two independent titrations. g) Size-exclusion chromatography (S200 10/300 GL) and SDS PAGE of tCAF-1•WTH3-H4 complex. The buffer contained 30 mM Tris pH 7.5, 100 mM NaCl, 1 mM EDTA, 1 mM TCEP. h) Schematic to show the binding stoichiometry of CAF-1 to different H3-H4 isoforms.



	measured MW (kDa)	
tCAF-1	-	145
tCAF-1-WTH3-H4 1:1	-	165
tCAF-1-XLH3-H4 1:1	296	153
	10.8 ml peak	12.2 ml peak

	theoretical MW (kDa)
tCAF-1	140
H3-H4	26
tCAF-1-H3-H4	166
(tCAF-1)2-(H3-H4)2	332

Figure 3.4 CAF-1 has a single binding site for a H3-H4 dimer (continued)

a) van Holde-Weischet analysis of SV-AUC runs performed with tCAF-1 and WTH3-H4, titrated in a 1:1 or 1:2 molar ratio of CAF-1 to H3-H4 dimer (orange and purple). A constitutively dimeric form of H3-H4, DMH3-H4 (green) was analyzed likewise. b) FRET-based Job plot assay with tCAF-1 and H3-H4 shows a single binding event with WT or DMH3-H4 dimers, while with XL(H3-H4)2 a stoichiometry of two CAF-1 complexes per tetramer is observed (total protein concentration was kept at 150 nM, where WT H3-H4 is dimeric). Two independent measurements are depicted; the data points are mostly overlapping. c) SEC-MALS experiment of tCAF-1 alone or in complex with WT or XL(H3-H4)$_2$. The sample containing XL(H3-H4)$_2$ shows an additional peak at ~300 kDa. The protein elution traces (refractive index, RI) refers to the right y axis, the calculated molar masses refer to the left y axis. Validation that tCAF-1 binds WT-H3-H4 on SEC is shown in Figure 3.3 g. d) Protection of regions on the H3-H4 dimer (from PDB: 1AOI) upon interaction with tCAF-1, based on HX-MS experiments (Figure 3.5). Red areas: regions with complete protection on both WT and DMH3 upon interaction with tCAF-1 (near 0 % uptake upon binding); orange regions: sites of intermediate HX protection upon interaction with tCAF-1. Blue and green indicate regions with no significant protection for H3 and H4 respectively. H3 αN is displayed with transparency because it is likely in a different conformation in the free H3-H4 dimer; no peptide coverage was observed for this region.

DMH3-H4 dimer, while two CAF-1 complexes are bound to one XL(H3-H4)$_2$ tetramer. This is observed for both FL and tCAF-1 (Figure 3.3f and 3.4b), supporting the idea that each CAF-1 complex has a single binding site for one H3-H4 dimer and confirming that the Cac1 N-terminus has no role in H3-H4 binding. Further confirmation for this stoichiometry comes from SEC-MALS (size exclusion chromatography coupled to multi-angle light scattering) experiments with tCAF-1, where significant increases in apparent molar mass are observed with XL(H3-H4)$_2$ but not with WTH3-H4, compatible with two tCAF-1 complexes binding to the XL(H3-H4)$_2$ tetramer (Figure 3.3g and 3.4c).

Altogether, these experiments indicate that each CAF-1 complex has a single binding site for one H3-H4 dimer, and that CAF-bound H3-H4 is still able to tetramerize, either with or without its own bound CAF-1 complex (Figure 3.3h). These assemblies are not stable enough to be isolated using WTH3-H4, but are observed with XL(H3-H4)$_2$. This suggests that the four-helix bundle formed by two H3 molecules in the (H3-H4)$_2$ tetramer is important for this complex formation and perhaps the only point of contact between the two CAF-1 moieties.

To further characterize the tCAF-1•H3-H4 complex, we used hydrogen-deuterium exchange coupled to mass spectrometry (HX-MS). CAF-1 binding to H3-H4 induces protection from exchange of an extended area on both WT and DMH3-H4, as opposed to a distinct region of protection. This suggests that CAF-1 binding to histones elicits a global conformational effect, which was also observed for other chaperone-histone complexes (D'Arcy et al., 2013; DeNizio et al., 2014) (Figure 3.4d and 3.5a). The most significant changes are clustered at the α1-α2 region of both histones (Figure 3.4d and 3.5a). This global conformational effect makes it difficult to identify the direct binding interfaces on H3-H4 (Figure 3.5b). Importantly, no significant changes in HX were detected in the H3 region mediating H3-H4 tetramerization through a four-helix bundle (α3; aa 111-126) (Figure 3.4d and 3.5a), confirming our interpretation that this region is exposed and available for tetramer formation in the CAF-1•H3-

H4 complex. Together, these data indicate that CAF-1 binds to one H3-H4 dimer that results in its overall stabilization, and that permits tetramerization in its CAF-1 bound form.

3.2.3 H3-H4 binding activates DNA binding by the Cac1 winged helix domain (WHD)

In our HX-MS experiments we observed HX changes distributed throughout all three CAF-1 subunits upon H3-H4 binding, with no direct evidence pointing towards a specific interface (Figure 3.5c). The observed HX changes are in agreement with recently published data from similar HX-MS experiments using the FL CAF-1 complex (Liu et al., 2016), confirming that deleting the N-terminal portion of Cac1 has no significant effect on histone binding. Moreover, by using both WT and DMH3-H4, we further support the idea that the mutations in DMH3-H4 do not affect its interaction with CAF-1 (Figure 3.5c). The broadly distributed HX changes suggest extensive structural rearrangements in the CAF-1 complex upon histone binding, with likely implications for the mechanism of histone deposition. In particular, we noticed that the C-terminal Cac1 WHD, recently identified as a DNA binding domain (Zhang et al., 2016a), showed deprotection upon histone binding in the HX-MS analysis (Figure 3.5c), also seen when FL CAF-1 was used (Liu et al., 2016). Interestingly, tCAF-1 does not bind DNA in absence of histones, despite containing a functional WHD domain (Figure 3.2b and below). We therefore asked if an intramolecular interaction between the histone binding region and the WHD may be masking the DNA binding activity of tCAF-1 in absence of histones. The acidic region, which constitutes part of the histone binding interface (Mattiroli et al, related manuscript; (Liu et al., 2016)), is a likely candidate for mediating this intramolecular interaction due to its complementary charges to the basic WHD. Indeed, mutant tCAF-1 complexes in which the Cac1 acidic region was neutralized or deleted, namely tCAF_Nac and tCAF_Δac respectively, gained DNA binding activity (Figure 3.6a-b, lanes 5-10). This indicates that in absence of histones the WHD may be inhibited by an intramolecular interaction with the acidic region on Cac1, a component of the histone binding module (Liu et al., 2016) together with Cac2

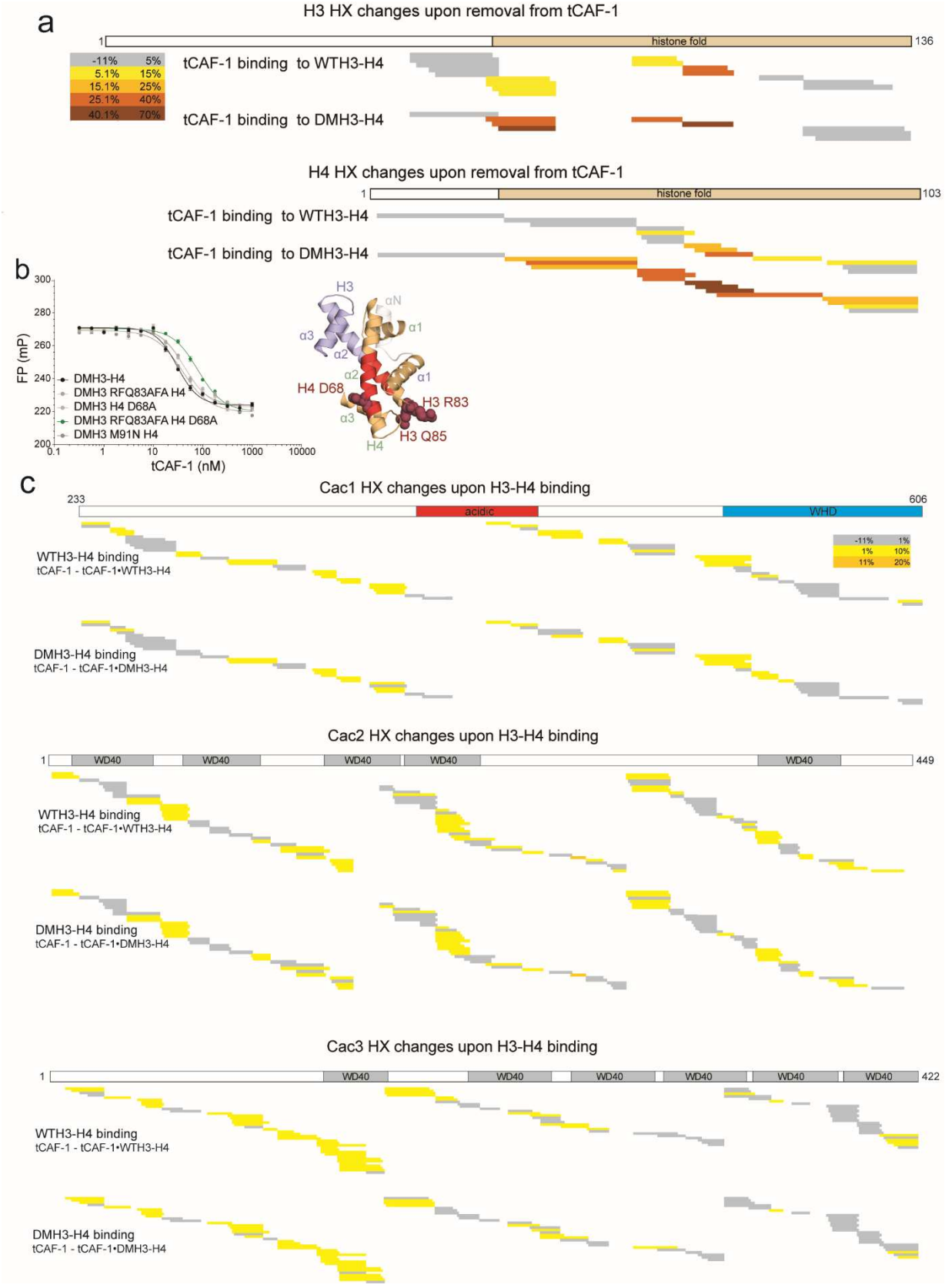


Figure 3.5 HX-MS data on CAF-1•H3-H4 complexes

a) HX-MS heatmap of the differences in deuterium uptake per H3 (top) or H4 (bottom) peptide when binding to tCAF-1 (60 minutes). The difference was calculated as percent uptake in free H3-H4 (unbound form) minus the percent uptake in tCAF-1•H3-H4 (bound form). Data related to WTH3-H4 histones is shown in the upper row; DMH3-H4 is shown below. The differences in uptake observed between WT and DMH3-H4 originate primarily from the fact that DMH3-H4 histones start less stably folded than the WTH3-H4 (DeNizio et al., 2014). CAF-1-bound DM or WTH3-H4 display highly similar deuterium uptake, although few peptides show differences, suggesting the possibility for minor dynamic changes on the histones upon CAF-1 binding (Supplementary File 1). b) Competition assay to measure the binding properties of H3-H4 mutants designed to interfere with the CAF-1 interaction. The probe was 10 nM tCAF-1●DMH3-H4 complex. The biggest difference was observed with DM H3 RFQ83AFA – H4 D68A (mutant residues mapped in dark red on the H3-H4 dimer structure on the right), which reduced the affinity 5-fold. Error bars show the SEM from three experiments. c) Heatmap showing the differences in deuterium uptake at 60 minutes for Cac1, Cac2 and Cac3 peptides analyzed in HX-MS experiments upon addition of either WT (top) or DM (bottom) H3-H4. The difference was calculated as percent uptake in tCAF-1 (unbound form) minus percent uptake in tCAF-1•H3-H4 (bound form). Supplememtary File 1 contains the data used to generate the HX-MS heatmaps. The deuterium uptake was not significantly different at shorter time points.

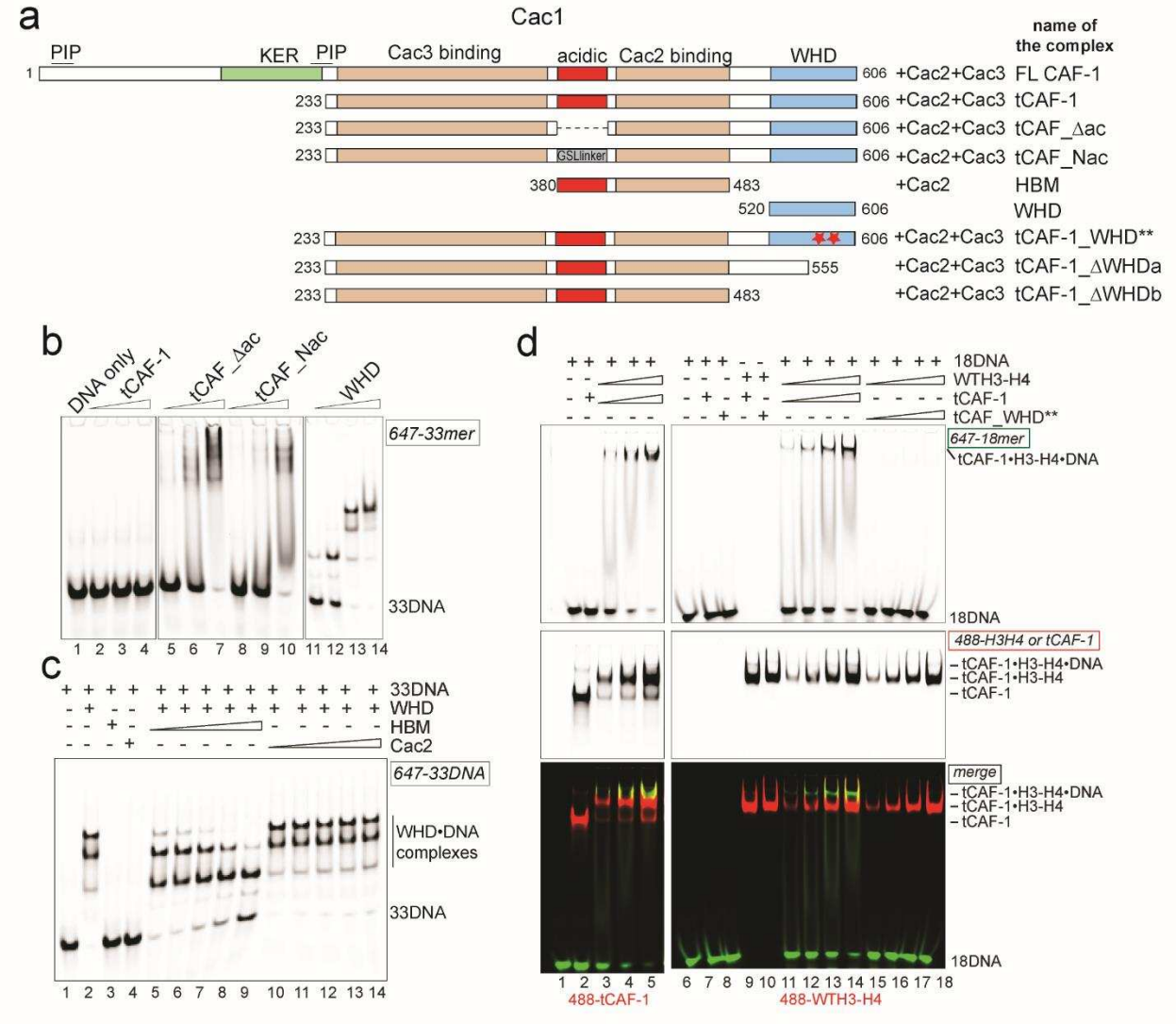


Figure 3.6 H3-H4 binding activates DNA binding by the CAF-1 complex

a) Schematic of the constructs and complexes used in this and subsequent figures. Cac1 domains that are combined with full length Cac2 and/or Cac3 as indicated, are depicted. PIP stands for PCNA binding peptide; tCAF_WHD** contains mutations at K564E and K568E. tCAF_Δac contains a deletion of residues 397-431 in Cac1, while in tCAF_Nac aa 397-431 were replaced with a Gly-Ser-Leu linker. KER identifies a Lys-Arg-Glu rich region. The Cac2 and Cac3 binding regions were mapped using HX-MS. Gel filtration profiles and SDS PAGE of these complexes are shown in Figure 3.7a. b) The acidic domain of Cac1 inhibits DNA binding by tCAF-1. 100 nM 33 bp DNA (33DNA) was mixed with tCAF-1, isolated WHD, or the tCAF-1 complexes mutated in the acidic region (depicted in a). CAF-1 complexes are titrated as 1-0.5-0.25-0.125 µM. c) The acidic domain competes WHD away from DNA. The WHD was kept constant at 1 µM and DNA at 100 nM. Cac2 and HBM were titrated as 4-2-1-0.5-0.25 µM. d) Histone binding releases the DNA binding activity of tCAF-1. EMSA performed with 100 nM 18 bp DNA (18DNA). tCAF-1 is titrated 0.37-0.75-1.5-3 µM, and was added either alone (in control lanes) or as a 1:1 complex with WTH3-H4 (H3-H4 dimer concentration) to the DNA. Alexa488-labeled tCAF-1 was used in lanes 1-5, while Alexa488-labeled H3-H4 were used in lanes 6-18).

(Mattiroli et al, related manuscript) (histone binding module is refered to as HBM, Figure 3.6a and 3.7a). We therefore performed a competition experiment by adding the HBM of CAF-1, or only Cac2 as control, to DNA-bound isolated WHD. Strikingly, the HBM could compete the WHD away from DNA, while Cac2 alone could not (Figure 3.6c). Moreover, using in solution cross-linking experiments we were able to trap the complex between the WHD and the HBM, a complex that contains the acidic region and Cac2, but not between the WHD and Cac2 (Figure 3.7b). Together, these data suggest that the Cac1 acidic domain engages in an inhibitory intramolecular interaction with the WHD, thereby masking its DNA binding capacity.

To directly test if H3-H4 binding can unmask the DNA-binding activity of the WHD, we established a gel-based DNA binding assay with tCAF-1 and histones. Here, pre-mixed stoichiometric tCAF-1•H3-H4 complexes were combined with limiting amounts of DNA and then analyzed on native gels to evaluate the formation of ternary tCAF-1•H3-H4•DNA complexes. We used an 18 bp DNA (18DNA) fragment, which represents the minimal substrate for binding to the WHD domain (Gajiwala et al., 2000), but is of insufficient length for wrapping the $(H3\text{-}H4)_2$ tetramer. This is important, as using longer DNA promotes rapid histone deposition and would therefore not allow us to demonstrate the DNA binding activity of histone-bound tCAF-1. As expected, tCAF-1 did not bind to 18DNA in absence of histones (Figure 3.6d, lane 2). However, when tCAF-1 was pre-incubated with histones, we observed a distinct high molecular weight complex, migrating slower than the tCAF-1•H3-H4 complex without DNA, and containing DNA, H3-H4, and tCAF-1 (Figure 3.6d, lanes 3-5 and 11-14). To confirm that DNA binding in this ternary tCAF-1•H3-H4•18DNA complex is mediated through the Cac1 WHD, we tested tCAF-1 complexes with a mutated WHD (tCAF_WHD**, containing Cac1 K564E K568E, Figure 3a). This mutant complex does not form the DNA-bound intermediate (Figure 3.6d, lanes 15-18), even though it still binds H3-H4 (Figure 3.6d, lane 10) with the same affinity and stoichiometry as tCAF-1 (Figure 3.7c-e). These data strongly suggest that H3-H4 triggers DNA binding by the

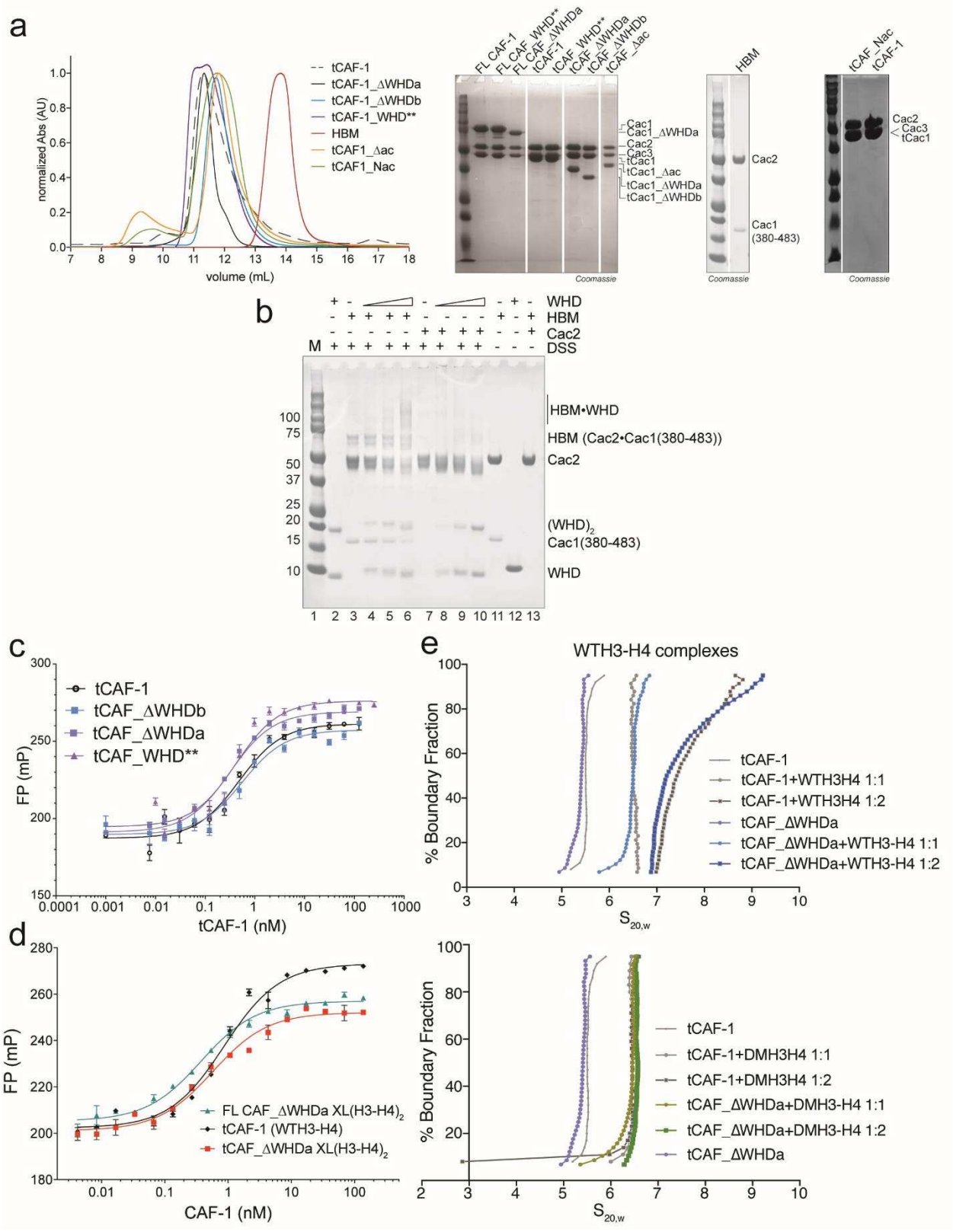


Figure 3.7 H3-H4 binding activates DNA binding by the CAF-1 complex (continued)

a) Gel filtration profiles (S200 10/300 GL column) for the complexes described in Figure 3.6 a. SDS PAGE analysis of the purified samples. The HBM sample is also shown on SDS PAGE in panel b lane 11. All the complexes do not show any aggregation and elute from gel-filtration at the expected volume for their size, indicating that the stoichiometry of the subunits is not altered. b) In solution cross-linking experiments with 5 µM of either HBM [Cac1(380-483)•Cac2] or Cac2 alone and variable amounts of WHD (0-5-10-20 µM). DSS was added at 2 mM and incubated for 30 minutes, before quenching and analysis by SDS PAGE. c-d) WHD mutation or deletion does not alter the affinity for H3-H4. Affinity measurements (FP) of the tCAF-1 complexes mutated or deleted in the WHD, to WT (c) or XL(H3-H4)2 (d) show no changes in Kd (Kd between 0.5-0.9 nM). Error bars depicts the SEM from three measurements. e) WHD deletion does not affect the stoichiometry of the CAF-1 interaction with histones. van Holde-Weischet analysis of SV-AUC experiments performed with tCAF_ΔWHDa and either WT (top panel) or DMH3-H4 (bottom panel) titrated as 1:1 or 1:2 ratio of CAF-1 to H3-H4 dimer. The curves also shown in Figure 2a obtained with the tCAF-1 complex are shown in gray as reference.

WHD, by releasing it from an inhibitory intramolecular interaction with the histone binding region.

3.2.4 DNA promotes the association of two CAF-1 complexes to form the $(H3\text{-}H4)_2$ tetramer

This ternary complex may well represent a relevant intermediate in the histone deposition mechanism of CAF-1. To determine the structural organization of the tCAF-1•H3-H4•18DNA complex, we investigated the effect of using a constitutively dimeric DMH3-H4 or a constitutively tetrameric XL(H3-H4)2 in this context. First, we wondered if a dimeric DMH3-H4 could form this ternary intermediate. Indeed, tCAF-1•DMH3-H4 assembles a similar complex with 18DNA to what was observed with tCAF-1•WTH3-H4 (Figure 3.8a, lanes 11-14) indicating that histone tetramerization is not required for the formation of this intermediate. This in turn also supports the idea that CAF-1 binds an H3-H4 dimer, and that binding to a histone H3-H4 dimer is sufficient to release the WHD and engage it in DNA binding. In addition, we wondered whether a constitutively tetrameric $XL(H3\text{-}H4)_2$ was still capable to assemble this intermediate. As shown in Figure 3.8b, tCAF-1•$XL(H3\text{-}H4)_2$ interacts with 18DNA forming the ternary tCAF-1•$XL(H3\text{-}H4)_2$•18DNA complex, hence XL(H3-H4)2 binding is also able to displace the Cac1 WHD. Because we have reported that two tCAF-1 complexes can bind to $XL(H3\text{-}H4)_2$ in absence of DNA (Figure 3.3f and 3.4b), we wanted to test whether this stoichiometry was maintained after DNA binding. To this end, we used tCAF-1 mutated in the WHD, in complex with $XL(H3\text{-}H4)_2$ (Figure 3.8c). We saw no interaction of (tCAF_WHD**)2•XL(H3-H4)2 with 18DNA, confirming that the Cac1 WHD is the primary point of contact to DNA and that both dimers in tetrameric $XL(H3\text{-}H4)_2$ are shielded from DNA binding. As 18DNA can interact with $XL(H3\text{-}H4)_2$ (Figure 3.8b, lane 4, demonstrated by disappearance of the free DNA band into the well), this strongly suggests that two CAF-1 moieties remain bound to the $XL(H3\text{-}H4)_2$ tetramer, otherwise the exposed H3-H4 dimer in the constitutive tetramer $XL(H3\text{-}H4)_2$ would remain free to mediate

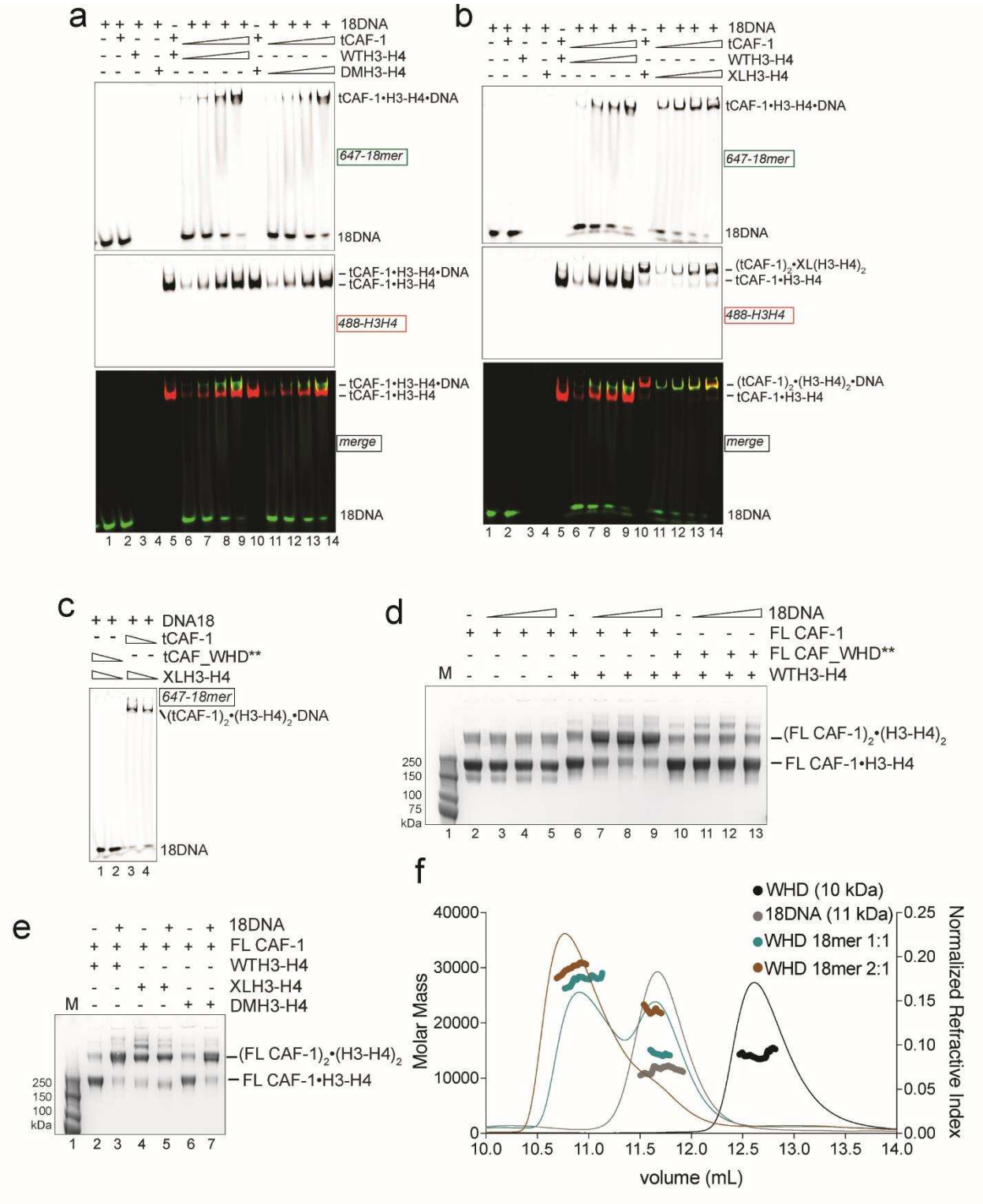


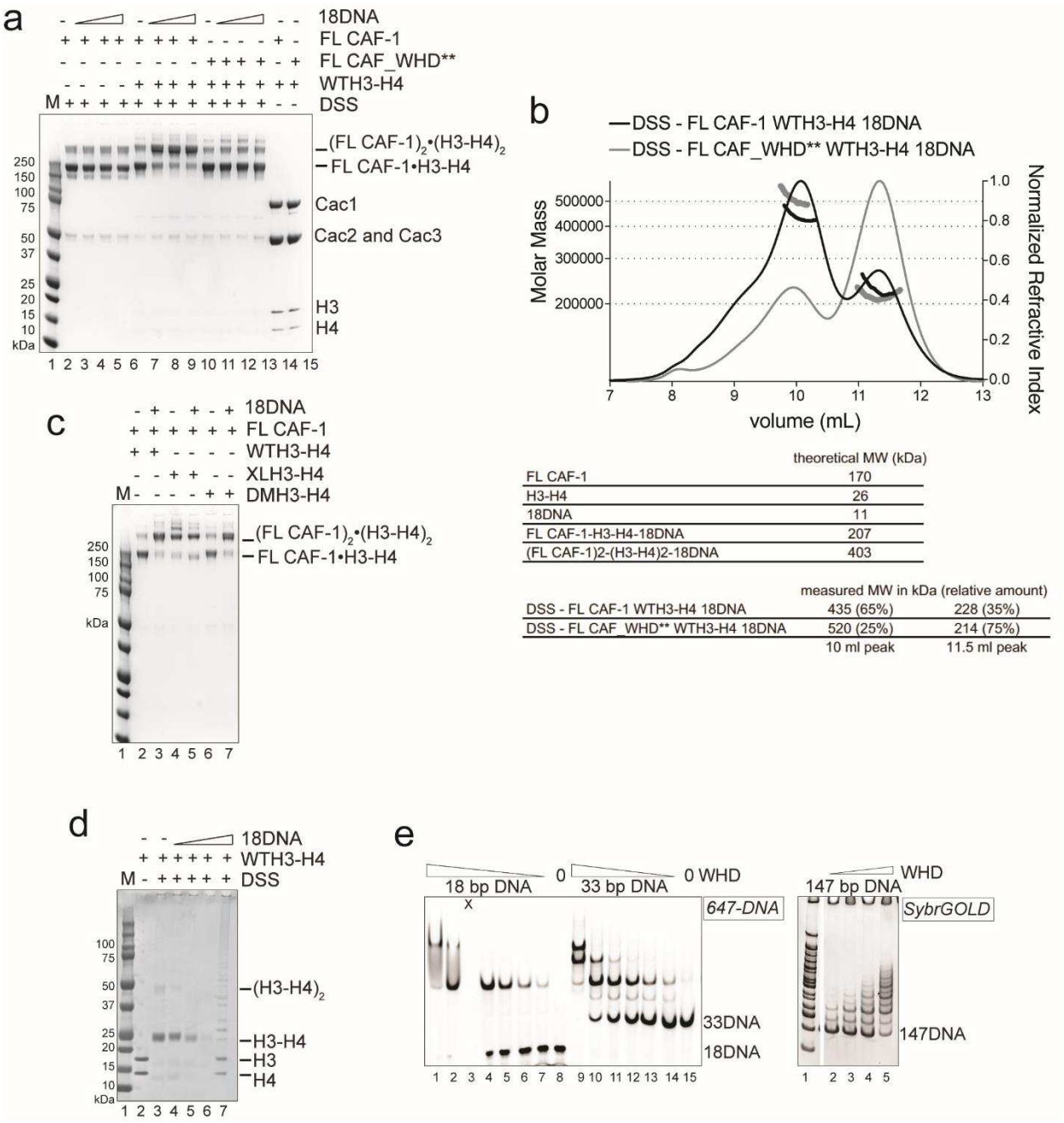
Figure 3.8 Two CAF-1•H3-H4 complexes assemble on a short DNA fragment.

a-b) A ternary tCAF-1•histone•18DNA complex can be formed with DMH3-H4(a) or with XL(H3-H4)2(b). EMSA was performed with 100 nM 18 bp DNA (18DNA). tCAF-1 is titrated 0.37-0.75-1.5-3 μM, and is added at a 1:1 ratio to either DMH3-H4 (a) or XL(H3-H4)2 (b). H3-H4 was

calculated as a dimer, even with XL(H3-H4)2 to the DNA. WTH3-H4 is shown as a control in both panels. c) Both H3-H4 moieties in the XL(H3-H4)2 are shielded from DNA binding in complex with tCAF-1. EMSA performed with 100 nM 18 bp DNA (18DNA). tCAF-1 or tCAF_WHD** in complex with XL(H3-H4)$_2$ [1 CAF-1 complex per 1 H3-H4 dimer] is at 1 and 3 µM. d) In solution cross-linking experiments with 2 µM of either FL CAF-1 alone, FL CAF-1•H3-H4 complex, or FL CAF_WHD**•H3-H4 complex, and variable amounts of DNA (0-1-2-4 µM). DSS was added at 1 mM and incubated for 30 minutes, before quenching and analysis by SDS PAGE. Full gel image shown in Figure 3.9a. e) In solution cross-linking experiments with DSS as in panel d, but here FL CAF-1 was premixed with either WT, DMH3-H4 or XLH3-H4. DNA is at 4 µM. Full gel image shown in Figure 3.9c. f) SEC-MALS experiment of the isolated WHD alone or in complex with 18DNA. In buffer containing 150 mM NaCl, the WHD is monomeric in absence of DNA, but on 18DNA it favors binding in a 2:1 stoichiometry (WHD to 18DNA). The protein/DNA elution traces (refractive index, RI) refers to the right y axis, the calculated molar masses refer to the left y axis.

DNA binding even with a complex with a mutated WHD. Because the tCAF-1•WTH3-H4•18DNA and the tCAF-1•DMH3-H4•18DNA complexes migrate similarly to the complex formed with XL(H3-H4)$_2$, which we know contains two tCAF-1 moieties (Figure 3.4b-c), and considerably slower than the tCAF-1•WTH3-H4 complexes without DNA (Figure 3.8b, lane 5-9, 488-H3-H4 red signal), we postulated that 18DNA promotes the association of two tCAF-1•WTH3-H4 moieties, and in this assembly histone tetramerization is not a requirement for complex formation.

To test this hypothesis, we performed in solution cross-linking studies. This allowed us to trap the assemblies without the potential artifact of the gel or column media, and also provided us with an opportunity to test our model with the FL CAF-1 complex, as the readout does not depend on DNA binding. Strikingly, titrating 18DNA into a FL CAF-1•WTH3-H4 complex strongly promoted the formation of a discrete high molecular weight assembly, which did not accumulate when the histones were omitted or when the Cac1 WHD was mutated (Figure 3.8d and 3.9a). By analyzing these reactions through SEC-MALS, we confirmed that this species has a molar mass consistent with the assembly of (CAF-1)$_2$•(H3-H4)$_2$•18DNA (Figure 3.9b). In line with the cross-linking data, this species was not favored when the WHD was mutated, and the most abundant assembly was composed of a single CAF-1•H3-H4•18DNA complex (Figure 3.9b). This association was further supported by monitoring the formation of this band with XL(H3-H4)2 and DMH3-H4, where a (CAF-1)$_2$•(H3-H4)$_2$ species is formed with XL(H3-H4)$_2$ even in absence of 18DNA (Figure 3.8e and 3.9c), as predicted by our binding stoichiometry data (Figure 3.3f, 3.3h and3.4c). Importantly, titrating 18DNA into H3-H4 in absence of CAF-1 did not significantly stimulate the formation of (H3-H4)2 tetramer, confirming that the association of two H3-H4 dimers under these conditions requires the presence of the CAF-1 chaperone (Figure 3.9d). Together, these data demonstrate that two FL CAF-1 complexes associate on a 18DNA via their WHD domains, but only do so when bound to histones. To conclusively confirm that an



	theoretical MW (kDa)
FL CAF-1	170
H3-H4	26
18DNA	11
FL CAF-1-H3-H4-18DNA	207
(FL CAF-1)2-(H3-H4)2-18DNA	403

	measured MW in kDa (relative amount)	
DSS - FL CAF-1 WTH3-H4 18DNA	435 (65%)	228 (35%)
DSS - FL CAF_WHD** WTH3-H4 18DNA	520 (25%)	214 (75%)
	10 ml peak	11.5 ml peak

Figure 3.9 Two CAF-1•H3-H4 complexes assemble on a short DNA fragment (continued)

a) In solution cross-linking experiments with 2 µM of either FL CAF-1 alone, FL CAF-1•H3-H4 complex, or FL CAF_WHD**•H3-H4 complex, and variable amounts of DNA (0-1-2-4 µM). DSS was added at 1 mM and incubated for 30 minutes, before quenching and analysis by SDS PAGE. Top part of this gel is shown in main Figure 3.8d. b) SEC-MALS experiment of FL CAF-1 or FL CAF_WHD** in complex with WTH3-H4 after DSS crosslinking treatment, as shown in Figure 3.8d. With an intact WHD, the main species in solution appear to have a molar mass consistent with two CAF-1•H3-H4 moieties. The protein elution traces (refractive index, RI) refer to the right y axis, the calculated molar masses refer to the left y axis. Note that DNA is not chemically cross-linked by DSS. c) In solution cross-linking experiments with DSS as in panel a,

but here FL CAF-1 was premixed with either WT, DMH3-H4 or XLH3-H4. DNA is at 4 µM. Top part of this gel is shown in main Figure 4e. d) In solution cross-linking experiments using WTH3-H4 at 2 µM and 18DNA titration at 0.5-1-2-4 µM. e) DNA binding assay of the isolated WHD (titrated 8-4-2-1-0.5-0.25 µM) to 18 or 33 bp DNA (1 µM) (left panel). X denotes a skipped lane. Right panel depicts WHD binding to 147 bp DNA (100 nM), the WHD was titrated (1-0.5-0.25-0.125 µM)

18DNA is sufficient to bridge two CAF-1 moieties, we measured the stoichiometry of the isolated Cac1 WHD when binding to 18DNA. In EMSA, we noticed that titration of an isolated WHD on longer DNA results in an alternate binding pattern that suggests a cooperative binding mode, while on 18DNA we only observed a single species (Figure 3.9e). SEC-MALS experiments demonstrate that this species contains two WHD domains, and that the binding of two WHDs to a 18DNA is preferred even at a 1:1 WHD to DNA stoichiometry (Figure 3.8f). Importantly, the Cac1 WHD elutes as a monomer on SEC-MALS in absence of DNA (Liu et al., 2016; Zhang et al., 2016a) (Figure 3.8f). This indicates that two Cac1 WHDs cooperatively bind DNA, as observed for other WHDs (Cornille et al., 1998; Gajiwala et al., 2000; Zheng et al., 1999) and suggest that this can bring together two CAF-1•H3-H4 complexes on 18DNA.

Overall, our data support a model in which the interaction between DNA and the WHD is triggered by histone binding to CAF-1, and this promotes the juxtaposition of two CAF-1 complexes, each pre-loaded with an H3-H4 dimer. Thus, the DNA-mediated association of two histone-bound CAF-1 complexes may promote the formation of the (H3-H4)2 tetramer on DNA.

3.2.5 DNA length promotes (H3-H4)2 deposition

How then is the (H3-H4)2 tetramer deposited onto DNA? Our data indicate that the H3 α3 helix in the H3-H4 dimers bound to CAF-1 is available for tetramerization with another H3-H4 dimer (Figure 3.4). Moreover, we show that two CAF-1 complexes bind on either side of a $(H3\text{-}H4)_2$ tetramer, and that an obligate H3-H3' interaction is sufficient to stabilize their association (Figure 3.4b), suggesting no additional significant contacts between the two CAF-1 moieties. Because of its limiting length, 18DNA only accommodates WHD binding, but cannot fully wrap the histone tetramer, which allowed us to trap the ternary complex. Because this complex can also be formed with dimeric DMH3-H4 (Figure 3.8a, lanes 11-14), we concluded that histone tetramerization is not required for the formation of the 18DNA-containing intermediate.

We next used longer DNAs to see if we could monitor subsequent steps in tetrasome formation, to understand how the two H3-H4 dimers are joined and to demonstrate that the (tCAF-1)2•(H3-H4)2•18DNA complex is relevant in the nucleosome assembly reaction. We used a 33 bp DNA (33DNA) which is longer than required for WHD binding, but still shorter than the ~70 bp necessary for complete wrapping of the $(H3\text{-}H4)_2$. tCAF-1 forms a ternary intermediate with WTH3-H4 and DNA, shown by EMSA (Figure 3.10a, lanes 1-6). As observed with 18DNA, its formation is dependent on the integrity of the Cac1 WHD (Figure 3.10a lanes 7-10 and 3.11a), supporting the idea that this domain mediates the primary interaction with DNA. Strikingly, the ternary complex containing 33DNA could not be stabilized with the constitutively dimeric DMH3-H4 (Figure 3.10b). This is surprising, as this histone mutant is sufficient to activate DNA binding by the WHD, as seen with the 18DNA (Figure 3.8a). These results demonstrate that histone tetramerization is required in this context. Hence, after the initial interaction via the Cac1 WHD, the formation of the ternary intermediate becomes dependent on the assembly of the $(H3\text{-}H4)_2$ tetramer with DNA of increasing length. This indicates that we are monitoring the step where the two H3-H4 dimers are joined to form the $(H3\text{-}H4)_2$ tetramer.

Consistent with the requirement for a histone tetramer, the ternary complex could be formed with XL(H3-H4)2 (Figure 3.10c). With this histone isoform, we noticed that the 33DNA ternary complex migrated faster on the native gel than the previously identified (tCAF-1)2•XL(H3-H4)2•18DNA intermediate, suggesting a significant change in molar mass (Figure 3.10c). Consistent with this, when using 33DNA with tCAF-1•XL(H3-H4)2, we saw a depletion of the peak corresponding to the (tCAF-1)2•XL(H3-H4)2 complex in SEC-MALS experiments (Figure 3.11b). Moreover, in cross-linking experiments with FL CAF-1 we noticed a similar trend, wherein the formation of a (FL CAF-1)2•XL(H3-H4)2•DNA complex was disfavored with 33DNA (Figure 3.10d, lane 5 and 3.11c). These observations support the idea that DNA of sufficient length partially destabilizes the CAF-1•histone interaction.

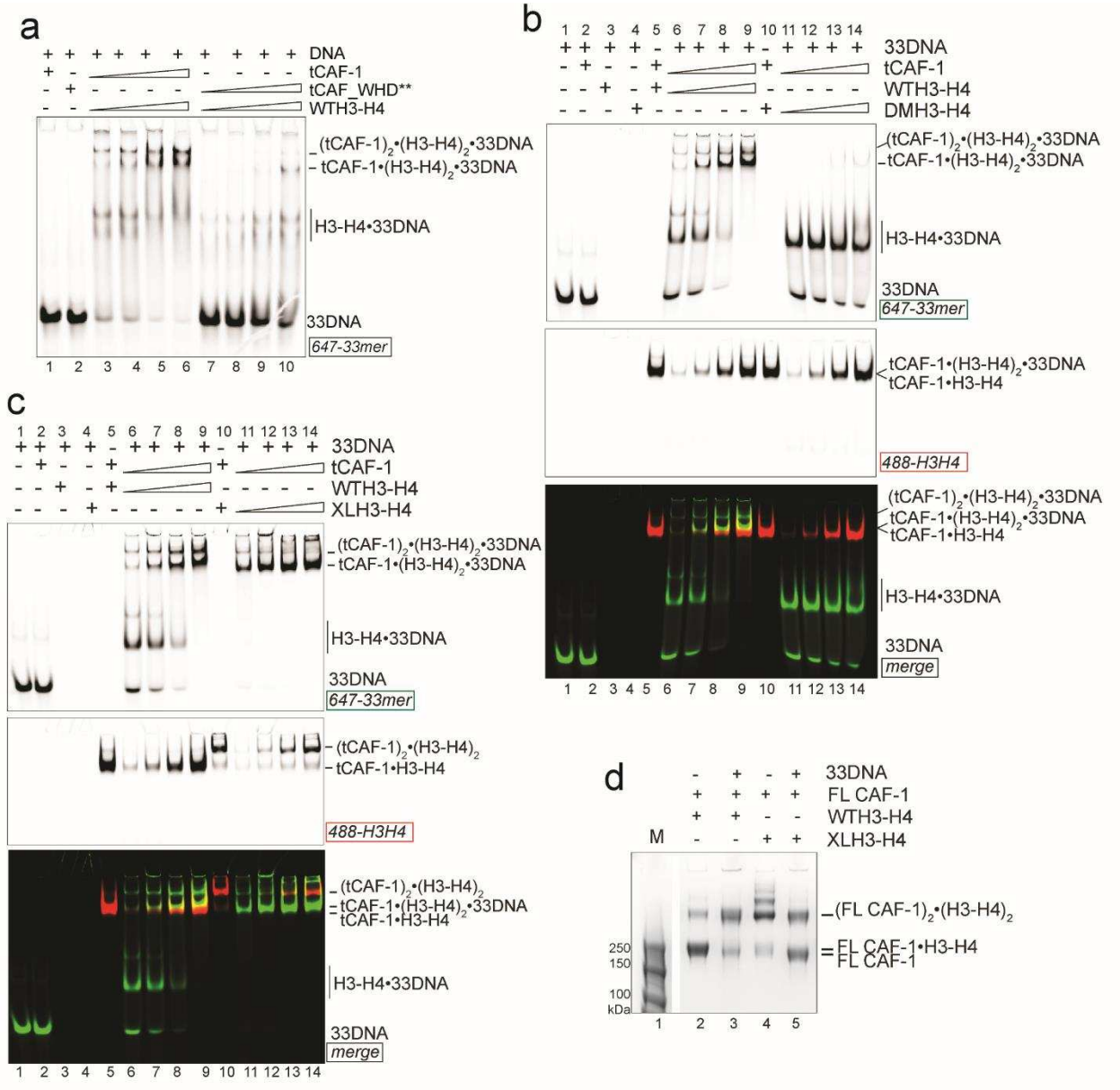


Figure 3.10 DNA of sufficient length sequesters $(H3\text{-}H4)_2$ from CAF-1

a) EMSA performed with 100 nM 33mer DNA. tCAF-1 or tCAF-1_WHD** were titrated 0.37-0.75-1.5-3 µM, and were added with WTH3-H4 to the DNA. b) EMSA performed with 100 nM 33 bp DNA (33DNA). tCAF-1 was titrated 0.37-0.75-1.5-3 µM, and was added with WT or DMH3-H4 (calculated as a H3-H4 dimer) to the DNA. c) EMSA performed with 100 nM 33 bp DNA (33DNA). tCAF-1 was titrated 0.37-0.75-1.5-3 µM, and was added with WT or XL$(H3\text{-}H4)_2$ (calculated as a H3-H4 dimer) to the DNA. d) In solution cross-linking experiments with 2 µM of FL CAF-1 premixed with either WT, DMH3-H4 or XLH3-H4, in presence of 4 µM of DNA. DSS was added at 1 mM and incubated for 30 minutes, before quenching and running of SDS PAGE. Full gel image is shown in Figure 3.11c.

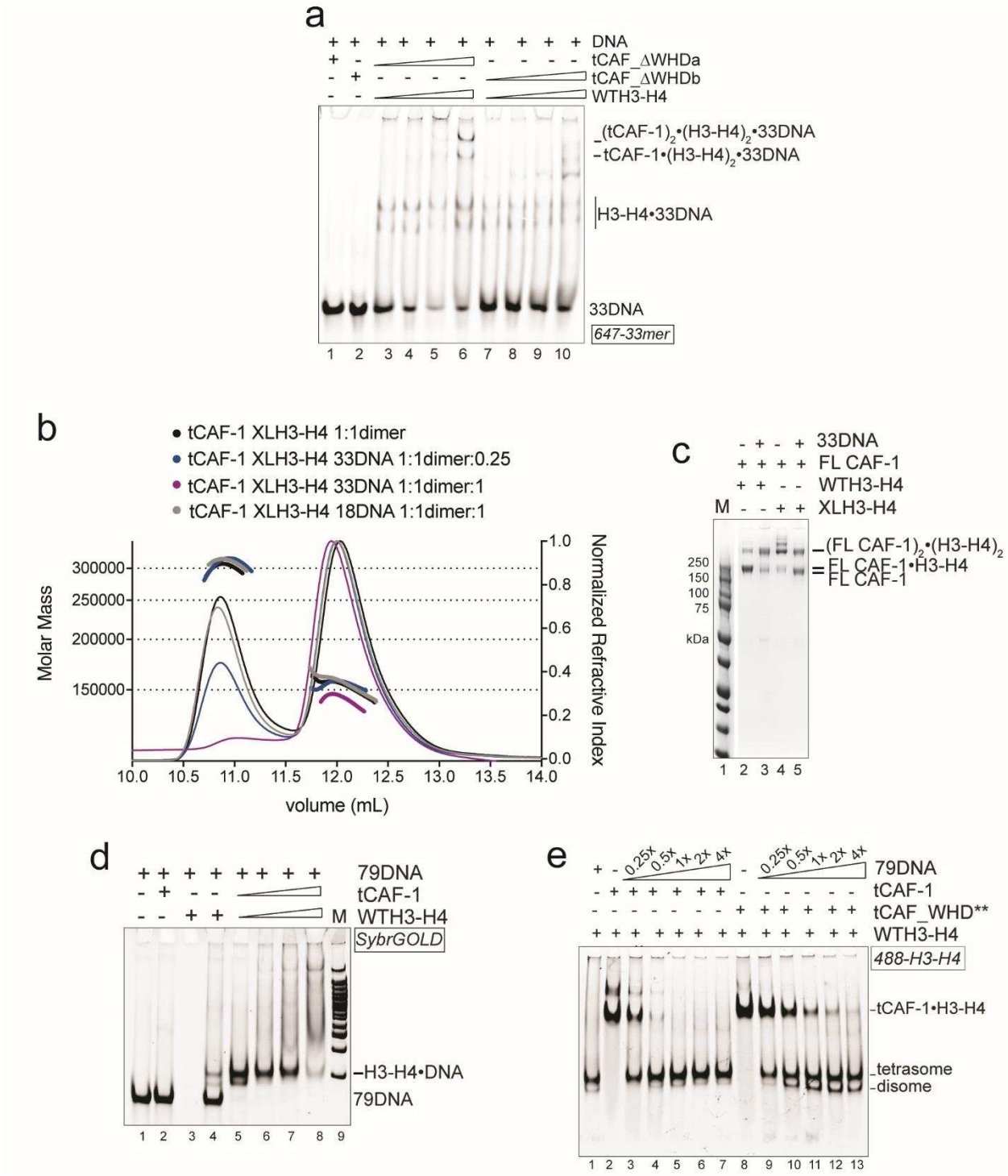


Figure 3.11 DNA of sufficient length sequesters (H3-H4)2 from CAF-1 (continued)

a) EMSA performed with 100 nM 33mer DNA. tCAF-1_ΔWHDa or tCAF-1__ΔWHDb were titrated 0.37-0.75-1.5-3 μM, and were added with WTH3-H4 to the DNA. b) SEC-MALS experiment titrating 33DNA into a tCAF-1 in complex with XL(H3-H4)2 (mixed as 1 CAF-1 per histone dimer). The sample with 18DNA is used as control. The protein/DNA elution traces (refractive index, RI) refers to the right y axis, the calculated molar masses refer to the left y

axis. c) In solution cross-linking experiments with 2 µM of FL CAF-1 premixed with either WT, DMH3-H4 or XLH3-H4, in presence of 4 µM of DNA. DSS was added at 1 mM and incubated for 30 minutes, before quenching and running of SDS PAGE. Top part of the gel is shown in main figure 5d. d) EMSA performed with 100 nM 79 bp DNA (79DNA). tCAF-1•WTH3-H4 is titrated 0.37-0.75-1.5-3 µM, to the DNA. e) EMSA performed with 200 nM tCAF-1•WTH3-H4 or tCAF_WHD**•WTH3-H4, 79 bp DNA (79DNA) is titrated at 25-50-100-200-400 nM.

Indeed, with a longer DNA (79 bp, 79DNA), suitable for complete tetramer wrapping, no intermediate could be formed and only the final assembly products, i.e. $(H3\text{-}H4)_2$•DNA complexes, are detected (Figure 3.11d). In this context, the Cac1 WHD is still required for efficient histone discharge from the histone chaperone, even in presence of excess DNA (Figure 3.11e).

Overall, our data point to a model for CAF-1-mediated tetrasome formation in which H3-H4 dimer interaction with the CAF-1 complex releases and activates the Cac1 WHD to allow its interaction with DNA (Figure 3.12a). This interaction promotes the association of two histone-bound CAF-1 complexes, via cooperative DNA binding of the Cac1 WHDs (Figure 3.12b). Here, the two CAF-1 complexes join their H3-H4 dimers to form a $(H3\text{-}H4)_2$ tetramer that can then be sequestered by the DNA tethered by the WHD (Figure 3.12c). The exact mechanism by which DNA promotes histone unloading from CAF-1 remains yet to be determined. We propose two hypotheses. In one case, the DNA remains partially bound to one WHD as it begins to wrap around the tetrasome, in another scenario the DNA dissociates from the WHD as it becomes bound to the (H3-H4)2 in proximity to the H3-H3' region, where it begins to pry the histones off CAF-1 (Figure 3.12c). The restoration of the inhibitory intramolecular interaction between the Cac1 WHD and the histone binding region, which likely occurs as the histones are unloaded from CAF-1, may also contribute to their transfer to the DNA molecule (Figure 3.12d). Together, our data suggests that the Cac1 WHD not only contributes to the mechanism by merely binding DNA, but by actively bridging together two CAF-1 bound H3-H4 dimers. Furthermore, by directly competing with the histone binding region, the WHD may facilitate the transfer of (H3-H4)2 from the chaperone to DNA.

3.2.6 The WHD dictates nucleosome assembly

Our model predicts that the integrity of DNA binding by the WHD is a pre-requisite for tetrasome formation and hence nucleosome assembly. Indeed, in the NAQ assay, tCAF-1 complexes with

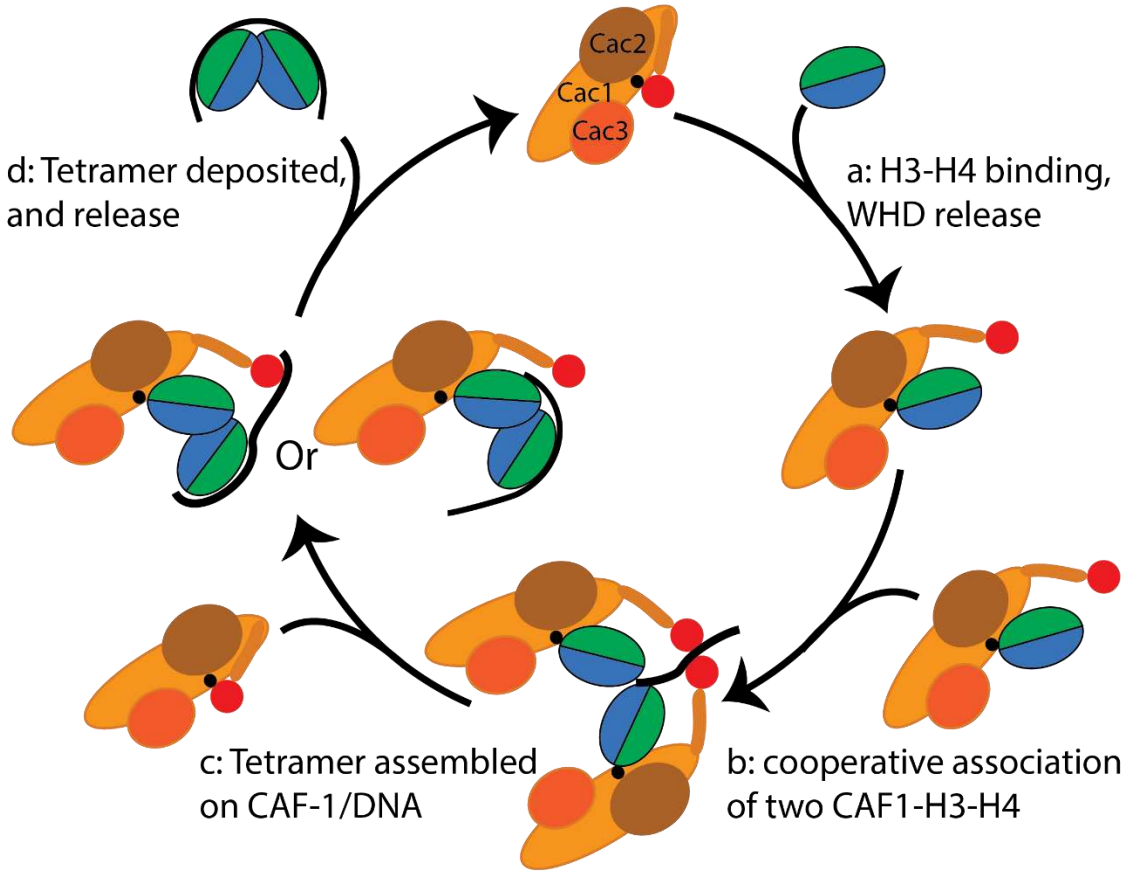


Figure 3.12 Model of the molecular mechanism of CAF-1 mediated tetrasome assembly.

a) The nucleosome assembly mechanism of CAF-1 is activated by H3-H4 binding, which releases the WHD domain from an intramolecular interaction with the acidic region on Cac1. b) DNA binding promotes the association of two CAF-1•H3-H4 complexes to join the histones into a $(H3\text{-}H4)_2$ tetramer. c) In the presence of DNA of sufficient length, the $(H3\text{-}H4)_2$ histones are directly sequestered from CAF-1. d) $(H3\text{-}H4)_2$ are transferred to the DNA to form the tetrasome, and the WHD re-binds to the now free acidic region, resulting in its dissociation from DNA.

a mutated or deleted WHD exhibit reduced nucleosome assembly activity (blue bars in Figure 3.13a). Notably, with these mutant tCAF-1 complexes we also observed a dose-dependent increase in protected DNA fragments around 100 bp rather than the 126-160 bp fragments observed with tCAF-1 and wild type CAF-1, indicative of the formation of subnucleosomal particles (Figure 3.13b). Similarly, in a native gel-based tetrasome assembly assay on either 147 or 79 bp DNA fragments, the WHD mutant complexes preferentially promoted formation of a band that migrates faster than the tetrasome (Figure 3.13c and 3.14a, disome). This band corresponds to the deposition of a single H3-H4 dimer onto DNA, as seen using the constitutively dimeric DMH3-H4 (Figure 3.13c). This finding indicates that either abrogating WHD-dependent DNA binding, or preventing H3-H4 tetramerization, result in the same deposition product *in vitro*, a single H3-H4 dimer bound to DNA. This fully supports our model in which $(H3\text{-}H4)_2$ tetramerization or deposition cannot occur in absence of CAF-1 DNA binding (Figure 3.12) and indicates a direct role for the WHD in regulating the fidelity of the nucleosome assembly reaction.

Surprisingly, when testing the effect of WHD mutation and deletion in FL CAF-1, we observed no difference in activity in the NAQ assay (Figure 3.13a). A possible explanation for this observation is that *in vitro* in absence of PCNA, the DNA binding property of the N-terminal part of Cac1 may compensate for the mutation of the WHD. We therefore moved to an *in vivo* system to address if the WHD is required for FL CAF-1 activity in the cell. We first used a previously described assay that monitors epigenetic silencing of a telomere-proximal URA3 reporter, a phenomenon which is dependent on the integrity of the Cac1 subunit (Kaufman et al., 1997). While WT Cac1 protein could completely rescue the growth phenotype on FOA plates, FL Cac1 WHD-mutants did not, reflected by delayed growth (Figure 3.13d and 3.14b). This indicates that the WHD domain is indeed important for Cac1 functions in the context of full-length Cac1 *in vivo*, in agreement with previous work (Zhang et al., 2016a).

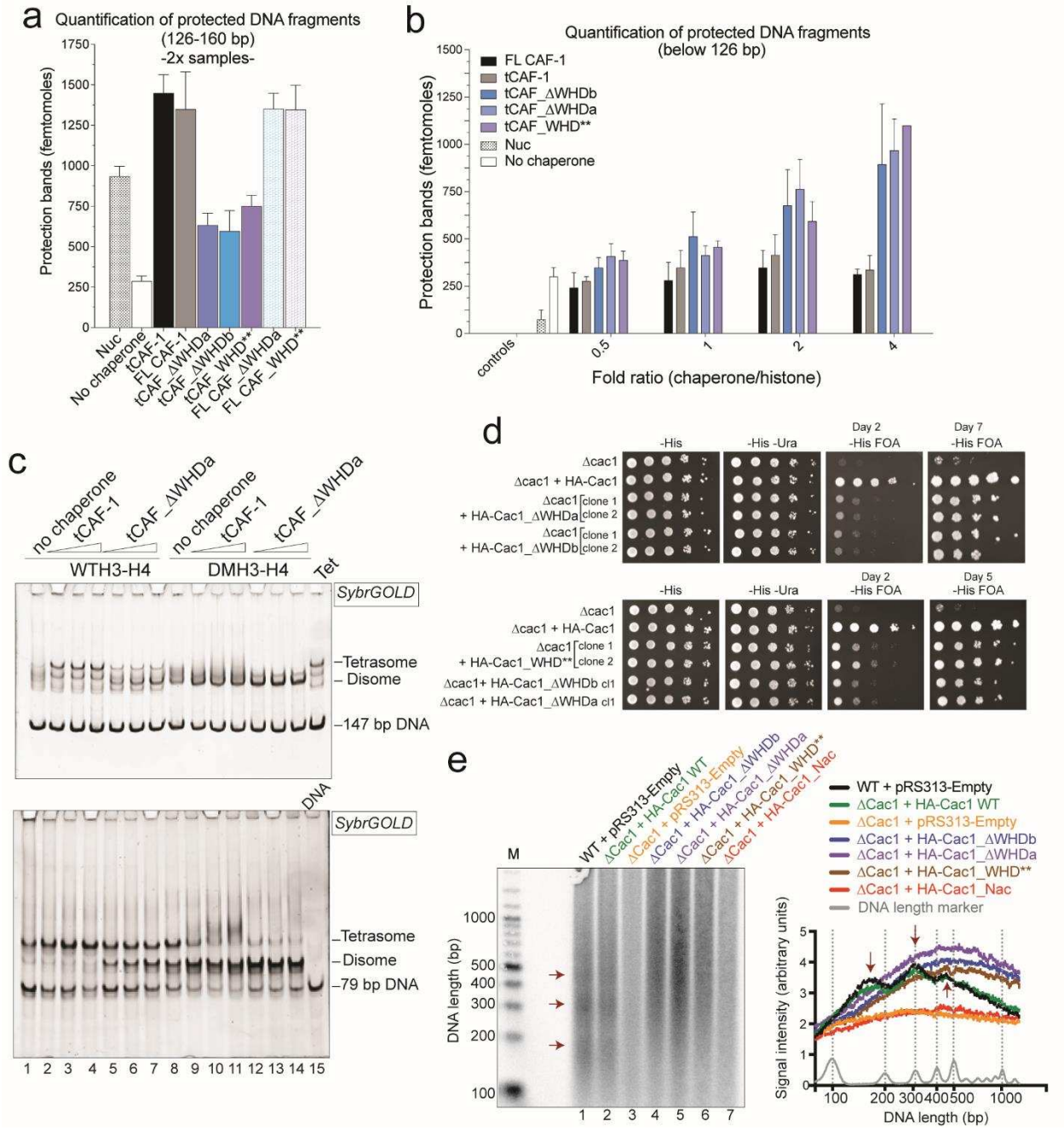


Figure 3.13 DNA binding by the Cac1 WHD is required for nucleosome assembly *in vitro* and *in vivo*.

a) Quantification of the nucleosome bands (126-160 bp) from the NAQ assays. Mean ± SD is shown from two or more independent measurements. For simplicity, we only show results from experiments with a 2-fold chaperone to histone ratio. The trends are identical for the 0.5-1-4 fold chaperone to histone ratio. The data use for this panel is included in Figure 3.13 Source Data 1 (https://doi.org/10.7554/eLife.22799.016). b) Quantification of bands below 126 bp (sub-nucleosomal species) from the NAQ assays. The error bars indicate SD from at least three repeats. The data use for this panel is included in Figure 3.13- Source Data 2 (https://doi.org/10.7554/eLife.22799.017). c) Tetrasome assembly assay on 147 bp (top

panel) or 79 bp DNA (bottom panel) with tCAF-1 and the tCAF_ΔWHDa mutant, comparing WT and DMH3-H4 deposition. Assembly for the other WHD mutants is shown in figure 3.14 a. d) Epigenetic silencing of a telomere-proximal URA3 reporter performed with yeast strains expressing the Cac1 mutation or deletion of the WHD. Samples were spotted at 0-101-102-103-104 dilutions from a OD600=1 stock. e) Okazaki fragment ends isolated from WT yeast strains, WHD mutant strains, and the acidic region mutant strain (Cac1_Nac, residues 397-431 were replaced with a Gly-Ser-Leu linker), as indicated, were radiolabeled and separated on a denaturing agarose gel. The right-hand panel depicts a normalized trace of signal intensity for each lane; DNA ladder is shown in light gray. Red arrows highlight the nucleosome-dependent length of the fragments in the positive control lanes (lane 1 and 2).

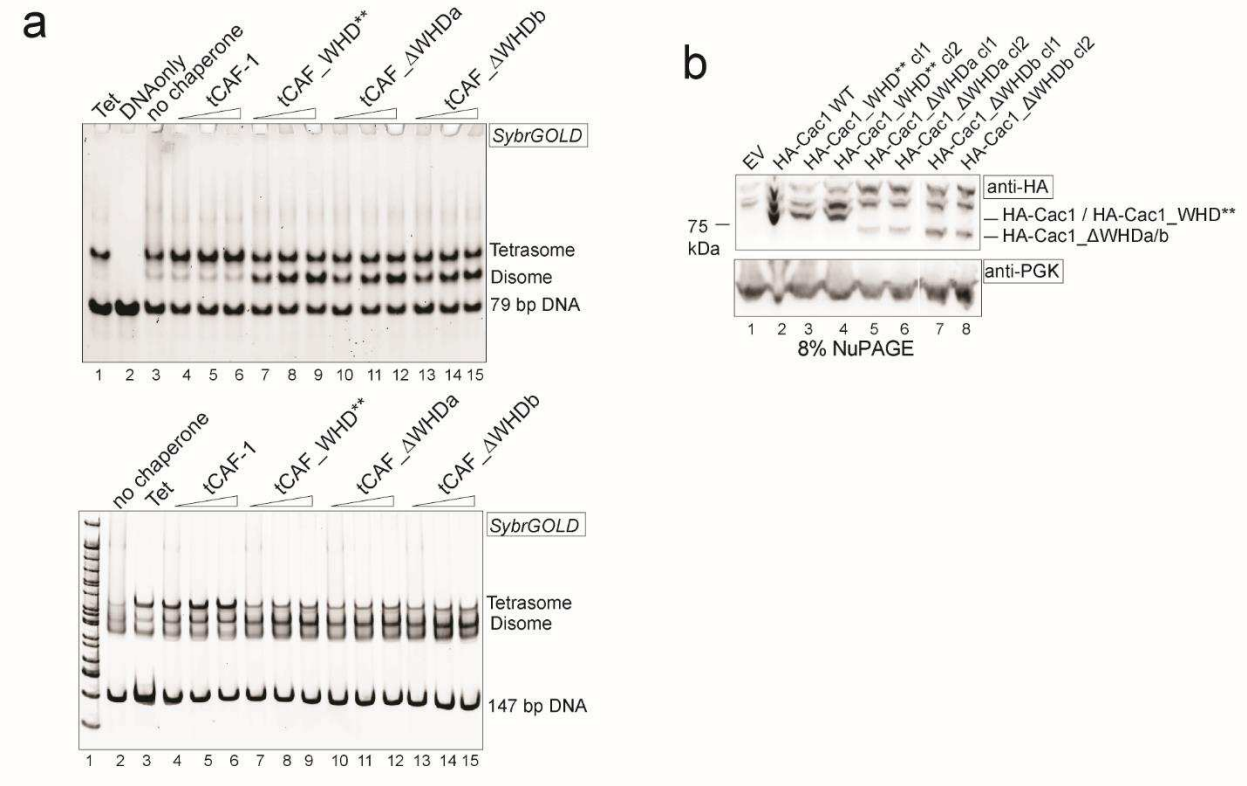


Figure 3.14 DNA binding by the Cac1 WHD is required for nucleosome assembly *in vitro* and *in vivo* (continued)

a) Cac1 WHD mutation or deletion result in the deposition of a H3-H4 dimer onto DNA. Tetrasome assembly assay with tCAF-1 WHD mutants on 79 (top) or 147 bp DNA (bottom). b) Cac1 mutants used in Figure 3.13d are expressed, as shown by immunoblot using anti-HA antibodies.

To specifically demonstrate that the phenotype observed with CAF-1 complex that is mutated in the WHD is due to an aberrant nucleosome assembly function of Cac1 *in vivo*, we took advantage of the fact that the length of Okazaki fragments generated during replication depends on proper nucleosome assembly by CAF-1 (Smith and Whitehouse, 2012; Yadav and Whitehouse, 2016). We analyzed Okazaki fragment length in yeast strains that carry mutations or deletions in the Cac1 WHD domain. Strikingly, Okazaki fragments purified from these strains do not exhibit the periodicity characteristic of a proficient replication-coupled nucleosome assembly pathway, but rather peak at length above 400 bp (Figure 3.13e). This phenotype is not as drastic as the one observed when we mutated the histone binding region (HA-Cac1_Nac, where the Cac1 acidic region is neutralized by a Gly-Ser-Leu peptide substitution; (Mattiroli et al., related manuscript). Yeast strains harboring this mutant CAF-1 complex produced an Okazaki fragment pattern that resembled that of the Cac1 knock-out (Figure 3.13e). Unlike mutation of the Cac1 acidic region, Cac1 WHD deletion and mutation do not affect histone binding (Figure 3.14c-e) and hence these mutant complexes, unlike HA-Cac1_Nac, are still able to recruit H3-H4 at the replication fork. We speculate that passive histone diffusion from these CAF-1 constructs to the DNA may occur, resulting in residual and inefficient histone deposition. The clear difference in Okazaki fragment formation between the WT and the WHD mutations confirms a deficiency in timely and accurate replication-coupled nucleosome assembly *in vivo*. These experiments show that the integrity of the Cac1 WHD is required for proper nucleosome formation on the lagging strand during DNA replication, fully supporting our model that places this domain at the heart of the CAF-1 nucleosome assembly mechanism (Figure 3.12).

3.3 Discussion

Combined, our data suggest a complex mechanism for the nucleosome assembly function of CAF-1 (Figure 3.12). We propose a model that involves DNA binding of two histone-bound CAF-1 complexes, thereby joining two H3-H4 dimers which are then transferred to adjacent

DNA, resulting in tetrasome assembly. Our data identifies the central role of the Cac1 WHD in orchestrating the assembly reaction through its histone-triggered DNA binding activity, and through promoting the association of two CAF-1 complexes, which culminates in (H3-H4)2 tetramer and ultimately tetrasome formation.

Our work reveals that two CAF-1 complexes are needed for the formation of one tetrasome, with each complex contributing a single H3-H4 dimer. This provides direct evidence for previously proposed models based on *in vivo* observations (Nakano et al., 2011; Tagami et al., 2004). Nonetheless, previous studies have suggested that one CAF-1 promotes $(H3\text{-}H4)_2$ tetramer formation, based on FRET measurements (Liu et al., 2012; Liu et al., 2016). Here we have studied the CAF-1•H3-H4 stoichiometry using a variety of techniques, including label-free methods such as AUC, SEC-MALS and HX-MS. Our data indicates that CAF-1 has a single histone binding site that interacts with a H3-H4 dimer, although a second H3-H4 dimer (either with or without its own CAF-1 complex) can associate with the CAF-1-bound dimer. We propose that this mode of binding is essential in the nucleosome assembly mechanism to allow the juxtaposition of the histone dimers to form (H3-H4)2 prior to deposition onto the DNA. This ability of CAF-1 to form different complexes with histones, depending on their concentration and conditions, may be responsible for the apparent differences in the literature (Liu et al., 2012; Liu et al., 2016).

We demonstrate the central importance of the WHD in the histone deposition process. A recent study has identified cross-links between the Cac1 WHD and histones (Liu et al., 2016). While this was interpreted as a direct interaction between the DNA binding domain and the histones, our data suggest that this may be due to the physical proximity between the WHD and the histone binding region (Figure 3.6b-d). This interpretation is also corroborated by a different study, where the WHD was found to cross-link with Cac2 (Kim et al., 2016), another important component of the histone binding interface on CAF-1 (Mattiroli et al., related manuscript).

Together, these findings suggest that the WHD remains in close proximity to H3-H4 on CAF-1. This could be an important structural aspect of the last step of the nucleosome assembly mechanism, where WHD-bound DNA sequesters the histones from the chaperone.

Our proposed model also demands that two CAF-1 complexes must be located in close proximity to be able to function together in tetrasome assembly. This implies that at least two CAF-1 complexes may simultaneously bind to one PCNA trimer. This is possible considering the presence of three potential binding sites for CAF-1 on a PCNA trimer, but will require further investigations. We cannot exclude that the interaction with PCNA may elicit additional structural rearrangement in CAF-1 that could control the nucleosome assembly mechanism. The importance of the cross-talk between the CAF-1 nucleosome assembly function and PCNA is supported by the finding that Cac1 WHD and PCNA binding mutants exhibit a synergistic effect *in vivo* (Zhang et al., 2016a).

Overall, our data suggest that a combination of relatively low affinity interactions (WHD•DNA, and H3•H3' in the H3-H4 tetramerization interface) work together to drive $(H3\text{-}H4)_2$ tetramer deposition onto DNA. This combinatorial effect ensures timely and accurate assembly reactions by bringing together chaperoned histones and DNA. Furthermore, on Cac1, the WHD is separated from the histone binding module by a flexible region (Mattiroli et al, related manuscript) which is post-translationally modified in a cell cycle dependent manner (Holt et al., 2009; Jeffery et al., 2015). These modifications may well play additional roles in fine-tuning the dynamic interactions of the WHD.

This study describes the mechanism of histone deposition by CAF-1, a reaction that has remained elusive for other histone chaperones. We demonstrate that an inhibitory intramolecular interaction poises CAF-1 for histone binding and deposition. H3-H4 dimer binding to CAF-1 activates the cascade of events required for its deposition, by unmasking the Cac1 WHD to bind DNA. This mechanism ensures that only histone-bound CAF-1 complexes are able

to participate in the deposition process, thus optimizing the efficiency of tetrasome assembly and excluding inefficient participation of empty CAF-1 complexes. By mutating the WHD, we uncouple histone binding and deposition, and demonstrate that high affinity histone binding is not sufficient to sustain efficient nucleosome assembly. This suggests that not all proteins that bind histones (i.e. histone chaperones) are in fact efficient nucleosome assembly factors.

Finally, the observation that two CAF-1•H3-H4 complexes associate to form a tetrasome suggests that two independently chaperoned H3-H4 dimers are joined at the very last step of the deposition process, when the DNA is in proximity and available for sequestering the histones from CAF-1. While convincing evidence supports the conservative model, where parental (H3-H4)2 are deposited in the same nucleosome following DNA replication (Xu et al., 2010), our proposed mechanism does not exclude the possibility of a semiconservative model for chromatin assembly coupled to DNA replication (Tagami et al., 2004). Further experiments are required to fully elucidate how CAF-1 complexes are paired in cells to understand the mechanisms underlying histone inheritance during cell division.

3.4 Materials and methods

3.4.1 Cloning and reagents

cDNA for the yeast CAF-1 complex were received from Paul Kaufman. These were cloned into the MultiBac vector for expression in insect cells. Cac1 was cloned into pACEBac1, Cac3 in pIDC, Cac2 in pIDS and these were recombined by Cre-Lox as described (Bieniossek et al., 2008). A His-tag was inserted at the C-terminal end of Cac2 for purification purposes. All complexes are prepared with a short C-terminal deletion of Cac2 (1-449), predicted to be disordered. Bacmids for expression in SF21 cells were prepared as previously described (Bieniossek et al., 2008). Mutations were introduced using Turbo Pfu polymerase (Roche) in a standard mutagenesis protocol. The Cac1 WHD cDNA was adjusted from the expression construct previously published

(Zhang et al., 2016a). The DNA used in the assembly and EMSA assays were based on the 601 Widom sequence and were prepared as previously described (Dyer et al., 2004).

3.4.2 Protein preparations

Xenopus laevis histones were purified from E.coli cells as previously described (Dyer et al., 2004), and stored in 2 M NaCl at -80°C. Labeling of histone proteins was performed as previously described (Muthurajan et al., 2016). Specifically, we labeled H4 with Alexa-488 on T71C, and H2B with AttoN-647 on T112C prior refolding with the histone partner. Constitutive dimeric DMH3-H4 is prepared as WT histones and contains H3 C110E L126A I130A. Constitutive XL(H3-H4)$_2$ tetramers were prepared by incubating 25 µM histone (H3 C110A K115C – H4 WT) in 20 mM HEPES pH 7.5, 1 M NaCl, 1 mM EDTA with 50 µM BMOE (bismaleimidoethane) for 1 hour at room temperature. Cross-linking was quenched with a 5x stock of DTT (final concentration 10 mM DTT and 10 µM XL(H3-H4)2). Cross-linking was assayed using a gel (samples were not boiled prior loading onto the gel).

CAF-1 was expressed in Sf21 cells and purified using a HisTrap column (GE) in buffer containing 50 mM TRIS 8.0, 600 mM NaCl, 5% glycerol, 10 mM imidazole, 5 mM BME (beta-mercaptoethanol), in presence of COMPLETE EDTA-free protease inhibitor (Roche), DNase I, 3 mM CaCl2 and 3mM MgCl2. The complex was then loaded on a MonoQ column in buffer A (50 mM TRIS 8.0, 200 mM NaCl, 1 mM EDTA, 1 mM TCEP) and eluted with buffer B containing 1M NaCl. The protein was then injected into a size exclusion column (Superdex 200) in 30 mM Tris pH 7.5, 300 mM NaCl, 1 mM EDTA, 1 mM TCEP. For HX-MS studies, buffer containing 50 mM KPO4, 150 mM NaCl, 5 mM DTT at pH 7.4 was used for the gel filtration step. Proteins were concentrated to 1-20 mg/ml and stored at -80°C in gel-filtration buffer. Mutants were purified as wild-type proteins; they behaved identically to wild type proteins during the purification procedure. CAF-1 complexes containing deletion or mutation of the acidic region in tCac1 were purified over a MonoS, instead of the MonoQ column with buffer A and B at pH 6.8. The WHD

domain was expressed as a GST fusion and purified according to published protocols (Zhang et al., 2016a).

3.4.3 Hydrogen Deuterium Exchange coupled to Mass Spectrometry (HX-MS)

CAF-1 complexes and/or histones to be analyzed by HX-MS were prepared at 4 µM in HX buffer (50 KPO4, 150 mM NaCl, 5 mM DTT pH 7.4). The CAF-1 complexes were gel filtered into the HX buffer, the histones were dialyzed overnight in the HX buffer to ensure that the final samples would not contain any additional buffer components that may result in buffer variability between proteins. HX reactions were set up by mixing 5 µl of the 4 µM stocks with 45 µl of deuterated HX buffer (prepared by dissolving in 99.9% D2O the lyophilized HX buffer) to result in a final 90% D2O concentration. Exchange was allowed to occur for 30 seconds, 1, 10, 30, or 60 minutes at 10ºC. Exchange was quenched by adding 50 µl of ice cold quench buffer (25 mM succinic acid, 25 mM citric acid at pH 2), that brought the sample to pH 2.4. Pre-quenched control reactions were prepared by adding quench buffer prior to D2O buffer. The samples were immediately injected into a temperature controlled (0ºC) Waters HDX Manager for online proteolysis at 12ºC using an Poroszyme immobilized pepsin column (Life Technologies), followed immediately by a 3 min simultaneous peptide trapping and desalting step at 0ºC using a Waters BEH UPLC C18 trap column, all with 100% solvent A (0.1% formic acid in water) flow at 100 µL/min. Peptides were then separated at 0ºC using a Waters 100 mm BEH C18 analytical UPLC column and a linear 8% to 40% solvent B (0.1% formic acid in acetonitrile) gradient over 6 min, followed by a 1 min 40% B hold and subsequent ramp to 85% solvent B in 0.5 min using a Waters nanoAcquity UPLC and 40 µl/min flow rate. The UPLC was coupled directly to a Waters Synapt G2 HDMS q-TOF mass spectrometer operating in positive, MSe data acquisition mode. Samples were incubated and analyzed in a random order. Non deuterated, prequenched, 1 and 60 minutes samples were taken in triplicate.

PLGS 3.0 (Waters) was used to create an identified peptide list from non-deuterated datasets and DynamX 3.0 (Waters) performed the search for deuterated peptide ion assignments. All isotope assignments for each peptide in each charge state were manually verified. The weighted average mass of each peptide determined by DynamX was then used to calculate deuteron uptake which was converted to % of deuteration based on the number of maximum exchangeable amide protons (number of total amino acids – Pro). Data were corrected for artefactual in-exchange using the quenched experiment as previously reported (Sours and Ahn, 2010). No corrections for back-exchange were conducted due to the comparison of relative uptake amounts between bound and unbound states, which would remain unaffected by the back-exchange correction. Graph bars and uptake plots were prepared using Microsoft Excel and GraphPad prism. The HX-MS uptake values of all the peptides analyzed is summarized in Supplementary File 1.

3.4.4 Tetrasome assembly assays

The assays were carried out in buffer containing 25 mM TRIS pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.02% Tween-20, 0.5 mM TCEP. CAF-1 was first diluted at different concentrations, normally a chaperone-histone ratio between 0.5 to 4 fold was used. Histones H3-H4 (100 nM tetramer concentration) were added, and the chaperone-histone mix was incubated at room temperature for 10 minutes. DNA was then added at 100 nM concentration. The reactions were incubated for 10-30 minutes (no differences were observed when incubating for longer time). Glycerol was added to a final concentration of 10% v/v prior loading of the samples on a 6% PAGE gel, pre-run in 0.2x TBE buffer at 4°C. The gels were run for 70 minutes at 150 V at 4°C. Gels were stained with SybrGOLD for 10 minutes and imaged on a Typhoon FLA 9500 at 488 nm.

3.4.5 NAQ assay (Nucleosome assembly and quantification)

The assembly assay was carried out as described above containing 200 nM of 207 bp DNA, 200 nM (H3-H4)2, 400 nM H2A-H2B and titration of CAF-1 (100-200-400-800 nM). After the assembly reaction, the samples were diluted to a DNA concentration of 50 nM in 100 µl digestion reactions. 25U of MNase enzyme was added in a final buffer containing 50 mM TRIS pH 7.9, 5 mM CaCl2. After incubation at 37°C for 10 minutes, the reactions were quenched with 10 µl of 500 mM EDTA, pH 8. The DNA was then purified using a modified protocol of the MinElute kit from QIAGEN. 550 µl of PB buffer and 10 ul of 3M sodium acetate were added to each sample and they were incubated at room temperature for 10 minutes. At this point, 50 ng of DNA loading control (or reference band, a 621 bp DNA fragment) was added to each tube. The samples were applied to the MinElute spin column and washed as prescribed by QIAGEN. The DNA was eluted with 10 µl of water. 1 µL of the eluate was used to load a DNA 1000 chip on the Bioanalyzer machine (Agilent), and 2.5 µl were loaded on a 10% PAGE gel. The gel was run for 45 minutes at 200 V in 0.5x TBE buffer at room temperature. Gels were stained with SybrGOLD for DNA and imaged on a Typhoon FLA 9500 (GE). The Bioanalyzer data was analyzed using the Agilent Expert 2100 software. The reference band was corrected for the proper size (621 bp) and the calculated molarity values were used to normalize all other bands present in the lane. The normalized values were used in the quantification and comparison. The signal threshold was set at 20 RFU. Nucleosome signal was calculated from bands ranging between 126-160 bp in length, based on the digestion of salt-reconstituted nucleosomes. Bands above or below this range were quantified separately. At least three independent repeats were performed per experimental condition.

3.4.6 Fluorescence Polarization experiments

Fluorescence Polarization assays were carried out in 25 mM TRIS pH 7.5, 300 mM NaCl, 5% glycerol, 1 mM EDTA, 0.01% NP-40, 0.01% CHAPS, 1 mM DTT (added fresh). Binding

reactions were prepared by mixing 5 nM of Alexa488-labeled H3-H4 dimer and increasing amounts of CAF-1. Binding data were measured using a BioTek Synergy H2 plate reader. The data was analyzed and plotted using Microsoft Excel and GraphPad Prism. The competition assay shown in Figure 1 – figure supplement 1e was done using fluorescence quenching rather than polarization. Representative curves are shown from one experiment (three independent measurement). The same conclusions were drawn from at least two other replicates, each containing three independent measurement per data point.

3.4.7 FRET-based stoichiometry experiments (Job plot)

FL or tCAF-1 were freshly labeled with an equimolar amount of AttoN-647 dye for 1 hour at 4°C. The reactions were quenched with 10 mM DTT. The protein was purified from the free dye on a PD-10 desalting column in buffer 20 mM TRIS pH 7.5, 300 mM NaCl, 1 mM EDTA, 1 mM TCEP. CAF-1 complexes were concentrated and mixed with Alexa488-labeled H3-H4 at constant total protein concentration of 150 nM, by inverse titrations of either species. Titrations containing only one labeled protein were used to correct the FRET signal. These were measured on a Typhoon or on a BMG ClarioStar plate reader. Dimer concentration was used for WT and DMH3-H4, while tetramer concentration was used for XL$(H3\text{-}H4)_2$. Representative curves are shown from one experiment (two independent measurement). The same conclusions were drawn from at least one other replicate, containing two independent measurement per data point.

3.4.8 Sedimentation Velocity Analytical UltraCentrifugation (SV-AUC)

SV-AUC experiments were carried out in a final buffer containing 20 mM TRIS pH 7.5, 300 mM NaCl, 1.6% glycerol, 1 mM EDTA, 1 mM TCEP. Samples were prepared at 4°C at a concentration of 4 µM CAF-1 (4 or 8 µM H3-H4 dimers) and run in a Beckman XL-A or XL-I centrifuges at 37000 rpm at 20°C. Absorbance at 280 nm was monitored. The data was analyzed using UltraScan III.

3.4.9 Size Exclusion Chromatography in line with Multi-Angle Light Scattering (SEC-MALS)

A Superdex 200 10/300 GL (GE) was mounted in line with a Optilab DSP and a DAWN Eos detectors (Wyatt). The runs were performed in 20 mM TRIS pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM TCEP at room temperature. 100 µL of protein sample at 15 or 100 µM (CAF-1 or WHD samples respectively) were injected at 0.4 ml/min, after being spun down at highest speed for 10 minutes. Data analysis was done using the ASTRA software (Wyatt) and GraphPad Prism was used to prepare figures. The WHD-18DNA experiments were run on a Superdex 75 10/300 column in line with a DAWN HELEOS II light scattering and a Optilab rEX refractive index detectors (Wyatt).

3.4.10 EMSA experiments

Ternary complex formation was set up in 25 mM TRIS pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.02% Tween-20, 0.5 mM TCEP. Proteins were incubated 10 minutes (3 µM of a 1:1 ratio of tCAF-1 and H3-H4 dimer concentration, or titration as described in figure legend) before addition of DNA (100 nM, titration of DNA to higher concentration didn't result in higher amount of ternary complex). Histone concentrations refer to histone dimers, even in the case of XL(H3-H4)2. 10% final concentration of glycerol was added before loading the samples into a 6% PAGE, pre-run in 0.2x TBE buffer at 150V. Gels were either scanned for fluorescence directly (AttoN647-labeled 18 or 33 bp and labeled histones) or first stained with SybrGOLD and then scanned on a Typhoon FLA 9500.

3.4.11 In solution cross-linking

2 µM of a 1:1 ratio of CAF-1 and H3-H4 dimer were incubated for 30 minutes in 20 mM HEPES pH 7.5, 75 mM NaCl, 1 mM DTT. Histone concentrations refer to histone dimers, even in the case of XL(H3-H4)$_2$. Then, DNA or buffer control was titrated at 1-2-4 µM (or just 4 µM for the single concentration reaction). After 10 minutes, 1 mM DSS (disuccinimidyl suberate) was

added and the reaction was left for 30 minutes at room temperature. The reactions were quenched with a final concentration of 70 mM TRIS pH 7.5. SDS loading buffer was immediately added and the samples were run on a NUPAGE 4-12% gel in MES buffer.

3.4.12 Yeast heterochromatin maintenance *in vivo* assay

The endogenous CAC1 locus was cloned into a pRS313 vector (the fragment contained 659 bp upstream and 731 bp downstream the Cac1 ORF). An HA-tag was introduced in the 5' end of the ORF. This construct was used to transform the PKY106 strain obtained from PD Kaufman (Kaufman et al., 1997). Mutants were generated using site-directed mutagenesis and were treated as the WT sample. The empty pRS313 vector was used as control. Transformed PKY106 strains were grown in synthetic media lacking histidine (-His). Clones were amplified overnight and then diluted in the morning and grown to an OD600 = 0.7-0.8. After washing the cells with water and then resuspending them to an OD600 = 1, four 10-fold dilutions from the initial stock were prepared. The undiluted sample and these dilutions were spotted on plates containing -His or -His-Ura media as controls, and -His supplemented with 1 mg/ml 5-Fluoroorotic Acid (FOA) for growth selection. Plates were left at 30°C for 2 days and then left at room temperature for up to the 7th day. To validate HA-Cac1 expression, 7 ml of cultures were harvested at OD600 = 0.7-0.8 and washed in water. The pellet was boiled for 3 minutes. 50 µl of PBS buffer containing 1 mM TCEP and COMPLETE EDTA-free protease inhibitors was added to the pellet. Cells were lysed using glass beads and the lysate was then spun down. The supernatant was loaded on a SDS PAGE gel and transfer to a PVDF membrane. The blot was probed with anti-HA antibodies (Abcam ab9110).

3.4.13 Okazaki fragment assay

Budding yeast strains yIW347 (wild-type, WT) and yIW397 (Δcac1) (Yadav and Whitehouse, 2016) carrying degron-tagged doxycycline-repressible alleles of CDC9 were transformed with empty vector pRS313. yIW397 was also transformed with pRS313,HA-Cac1 construct

described above. All transformants were verified and maintained by growth on YC-HIS minimal media. For the Okazaki fragment preparation, all strains were grown at 30°C in YC -HIS liquid medium supplemented with 2% glucose. 50 ml cultures were used for labeling experiments. At OD600 ~0.3, cells were harvested and re-suspended into 50 ml YEP medium and doxycycline was added to final concentrations of 40 mg/l and the culture shaken at 30°C for 2.5 hr. Genomic DNA was prepared from spheroplasts, radio-labeled, and visualized as described previously (Smith et al., 2015).

CHAPTER 4: TETRAMERIZATION OF H3-H4 IS A PREREQUISITE FOR CHAPERONE MEDIATED NUCLEOSOME ASSEMBLY

Overview

The eukaryotic genome is packaged in a chromatin complex with histones. The minimal unit of chromatin is the nucleosome, which consists of a histone octamer wrapped by 147 bp DNA in 1.7 turn of superhelix. The nucleosome represents a barrier for the accessibility of DNA on the nucleosome. The nucleosome is dynamic under circumstances such as transcription or DNA repair, during which histone H2A-H2B dimers will be depleted. However, the remaining $(H3\text{-}H4)_2$-DNA complex (the tetrasome) is a stable intermediate. In this study, we demonstrate that the tetramerization interface is the key for this high stability, and is essential for chaperone-mediated nucleosome assembly. Breaking the $(H3\text{-}H4)_2$ tetramers disables proper tetrasome assembly. However, we found that H2A-H2B dimers could behave as stabilizers for the unstable $(H3\text{-}H4)_2$ tetramers on DNA, indicating that H2A-H2B could be a regulatory target for destabilizing histone octamer and initiation of nucleosome disassembly.

4.1 Introduction

The eukaryotic genome is packed into chromatin, consisting of histones and DNA. The basic unit of this chromatin structure is the nucleosome, which is composed of a histone octamer (an $(H3\text{-}H4)_2$ tetramer and two H2A-H2B dimers) wrapped by 147bp DNA (Luger et al., 1997). Nucleosomes represent natural barriers for DNA-related processes, such as replication and transcription (Carey et al., 2006; Gaykalova et al., 2015; Gruss et al., 1993; Randall and Kelly, 1992; Workman, 2006). Therefore, nucleosomes must undergo dynamic re-arrangements through a network of histone chaperones, remodelers, histone modifiers, and transcription factors (Gurard-Levin et al., 2014; Mellor, 2005; Talbert and Henikoff, 2016; Tessarz and

Kouzarides, 2014). For example, the histone chaperone FACT is shown to organize nucleosome structure by destabilizing H2A-H2B dimers from the nucleosome, while maintaining the $(H3\text{-}H4)_2$ tetramer in a manner that allows for RNA polymerase II to traverse without splitting two H3-H4 dimers (Hsieh et al., 2013; Tsunaka et al., 2016). While the histone chaperone NAP1 is involved in preserving nucleosomes after the traverse of RNA polymerase II during *in vitro* transcription (Kuryan et al., 2012).

The structural organization of the nucleosome shows that the $(H3\text{-}H4)_2$ tetramer is centered in the core of the nucleosome, and that H2A-H2B dimers are docking on the both faces of the disc-like tetramer-DNA complex (tetrasome) (Luger et al., 1997). The histone octamer, which contains two copies of each H3, H4, H2A and H2B, can be reconstituted by dialysis of the mixture into high salt (2M NaCl), in which $(H3\text{-}H4)_2$ tetramers are assembled followed by cooperative incorporation of two H2A-H2B dimers to assemble into hexamer and octamer (Benedict et al., 1984). Histone octamers are vulnerable under physiological conditions, which are characterized by low ionic strength, suggesting that the hydrophobic interactions between histones play a dominant role for the stability of the octamer (Luger et al., 1997). However, the nucleosome is a highly stable protein-DNA complex under physiological conditions, with increasing dynamics as salt increases (Gansen et al., 2009). Therefore, histone octamer assembly is not the single driving force for the stability of nucleosome. Indeed, the histone octamer has intensive interactions with DNA through electrostatic interactions as well as numerous water-driven hydrogen bonds (Davey et al., 2002), and nucleosome positioning is strongly affected by the DNA sequence (Struhl and Segal, 2013). The assembly of nucleosomes in low salt is contradictory to the preference of high salt for histone octamer formation, suggesting that DNA plays an essential role to shape the histone octamer structure and prevent it from falling apart under physiological salt conditions. Therefore, nucleosomes can be reconstituted by gradually decreasing salt concentration *in vitro* as described before (Dyer et al.,

2004), in which the process, the $(H3\text{-}H4)_2$ tetramers are initially associated with DNA, finished by incorporation of two H2A-H2B dimers, similar to the histone octamer assembly (Burton et al., 1978; Rippe et al., 2008; Tatchell and Van Holde, 1977). While the salt-reconstitution mechanism of the nucleosome is well established, how this mechanism is related to the chaperone-mediated assembly is not clear.

High concentrated (in μM) H3-H4 dimers assemble into a tetramer in high salt conditions, with the help from hydrophobic interactions within the four helix bundle formed by H3-H3' (Eickbush and Moudrianakis, 1978). Therefore, under physiological salt conditions or even at lower salt concentrations, $(H3\text{-}H4)_2$ tetramers start to fall apart and exist in an equilibrium between dimers and tetramers (Banks and Gloss, 2004; Baxevanis et al., 1991; Donham et al., 2011). Moreover, histone chaperone Asf1 binds to H3-H4 *in vivo* in a manner that excludes the tetramerization of two H3-H4 heterodimers, indicating that histones H3-H4 are transported in a dimer form during the chromatin dynamics (English et al., 2006; Natsume et al., 2007). Interestingly, H3-H4 heterodimers are shown to cooperatively associate with DNA at high salt concentrations (~1.35M) (Burton et al., 1978), thus, it would be intriguing to test if tetramer assembly prior to deposition is required for nucleosome assembly. In absence of DNA, H2A-H2B binds to tetramers in high salt condition, but with lower affinity than H3-H3' interaction (Eickbush and Moudrianakis, 1978). However, when a stable H3-H4-DNA complex is formed, H2A-H2B binding is favored even at low salt conditions (Burton et al., 1978), consistent with the two-step nucleosome assembly pathway. Each H2A-H2B dimer binds two moieties of H3-H4 dimers in the nucleosome through the H4-H2B four helix bundle and H2A docking region (Luger et al., 1997), but how these interactions contribute to the stability of the nucleosome is unclear. H2A-H2B has been known as one of the targets for regulating nucleosome dynamics, as it is located at the nucleosome DNA entry and exit sites. Multiple studies have shown that H2A-H2B is

displaced in the first stage of nucleosome disassembly (Gansen et al., 2009; Taguchi et al., 2014), suggestive of a potential target for trans-acting factors involved in this process.

In this study, we used a constitutive H3-H4 dimer mutant to test the stability of the tetrasome and nucleosome, showing that tetramer formation is required for stable tetrasome assembly, but not for nucleosome assembly during salt reconstitution. Interestingly, de novo nucleosome assembly by CAF-1, which is accomplished under physiological salt conditions, requires a stable tetramerization interface between two H3-H4 dimers (Mattiroli et al., 2017b), suggesting that stable tetrasome assembly is the prerequisite for de novo nucleosome assembly. Therefore, there are different mechanisms for nucleosome assembly between salt reconstitution and chaperone-mediated pathways. Moreover, further thermal stability experiments by using H2A docking site mutations or alternative DNA sequences suggest that H2A-H2B facilitates nucleosome stability by tethering two H3-H4 heterodimers within the nucleosome core, with the assistance of DNA.

Our studies of nucleosome assembly pathways, and the protein-protein interactions stabilizing the nucleosome advance our understanding of the regulation underlying nucleosome dynamics during DNA-related processes in cells.

4.2 Results

4.2.1 The tetramerization interface is required for histone octamer assembly in absence of DNA

Two histone H3-H4 heterodimers form a tetramer within the nucleosome, but this tetramer form is unstable under physiological conditions. The H3-H4 histones exist as an equilibrium between dimers and tetramers. Moreover, the histone chaperone Asf1 binds to H3-H4, in a manner that blocks the tetramerization interface of H3-H4, arguing that $(H3\text{-}H4)_2$ tetramer are vulnerable species when isolated from the nucleosome context. However, it is unclear if this interaction is required for histone octamer assembly, as H2A-H2B in the nucleosome binds two H3-H4

heterodimers through the H2A docking site and H4-H2B four helix bundle, while also maintaining L2-L2' interactions of two H2A-H2A' (Luger et al., 1997). To answer this question, we designed mutations in histone H3 that assemble into a constitutive dimer with wild type H4 (H3 C110E, L126A and I130A, hereafter DM H3-H4) (Figure 4.1c). These mutations break down the four helix bundle interface (Banks and Gloss, 2004). In a gel filtration purification step using 2M NaCl buffer, this dimeric mutant, when refolded with histone H4 forms a homogenous peak, is similar in elution volume to the H2A-H2B dimer peak, and much later than WT H3-H4 (Figure 4.1a), indicating that this mutant is mainly in a dimeric form even under conditions suitable for tetramer assembly. SDS-PAGE analysis of the peak fractions shows that it contains both histones at equimolar concentrations (Figure 4.1 b), and demonstrates that the peak shift is not caused by a partial degradation of this mutant. Interestingly, when I refolded DMH3-H4 together with H2A-H2B dimers, I did not see a peak corresponding to the histone octamer (Figure 4.1c and 4.2a). This data shows that a stable tetramer is required for histone octamer assembly at 2 M salt.

DSS crosslinking experiments demonstrate that histones are mainly in a dimeric form in solution (DMH3-H4 or H2A-H2B) (Figure 4.2b), while the crosslinked histone octamer should run at ~100 kDa based on previous observations (Vestner et al., 2000). These findings suggest that even though H2A-H2B dimers bind to two H3-H4 dimers within the histone octamer (Luger et al., 1997; Ramakrishnan, 1997), it could not stabilize a histone octamer in the absence of a stable H3-H3' interaction, highlighting that $(H3\text{-}H4)_2$ tetramerization is essential for proper histone octamer assembly.

4.2.2 Tetramerization interface is the key for stable tetrasome assembly

Having shown that the tetramerization interface is important for histone octamer assembly, we wanted to know if it is required for the assembly of the tetrasome, in which two H3-H4 heterodimers are assembled in a tetramer form onto DNA. Tetrasome assembly is the initial

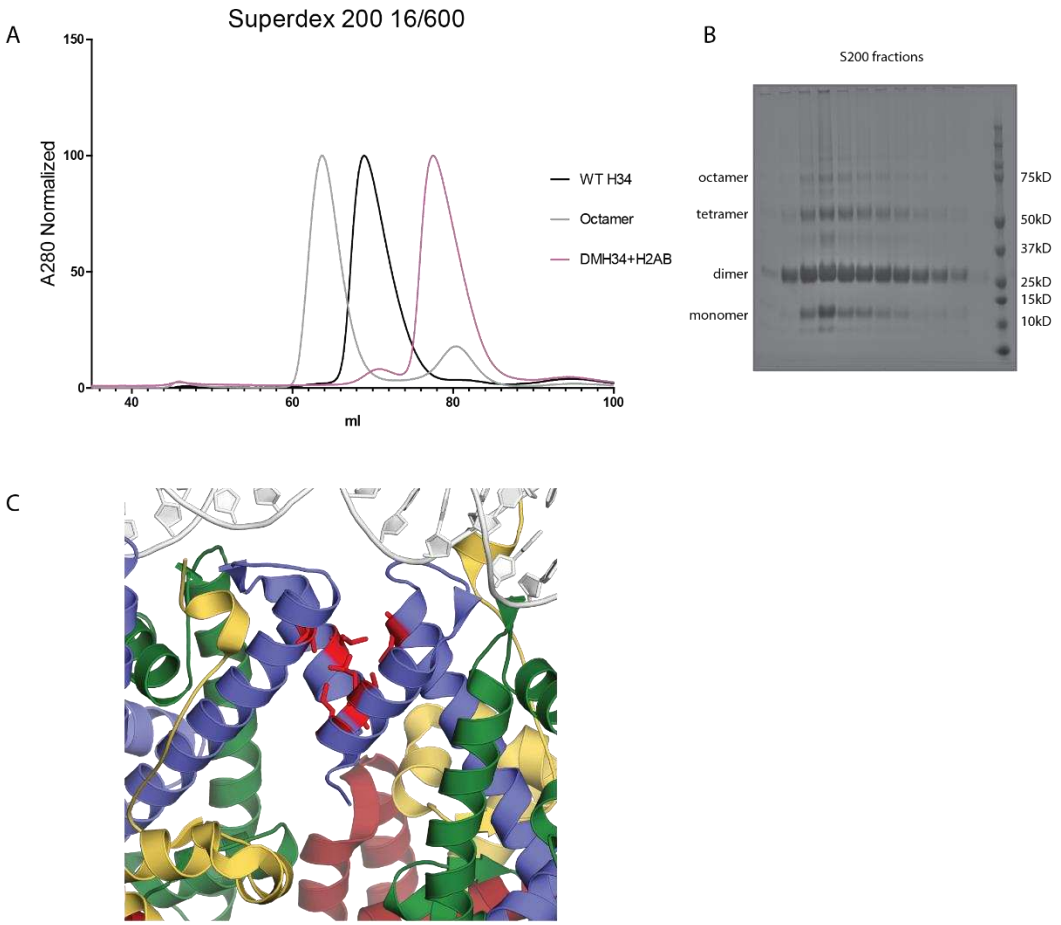


Figure 4.1: H3-H3' interface is important for proper histone octamer assembly

A.) Gel filtration chromatography of refolded histone complexes using 16/600 Superdex 200 column. WT H3-H4 (black) represents the peak position for a tetramer. The A280 absorbance of each sample was normalized for comparison. B.) Crosslink of DMH3-H4+H2A-H2B peak samples by DSS, analyzed on SDS-PAGE. C.) The mutated positions in DM H3 (Shown as stick in red) (PDB:1kx5). H3 is colored in blue.

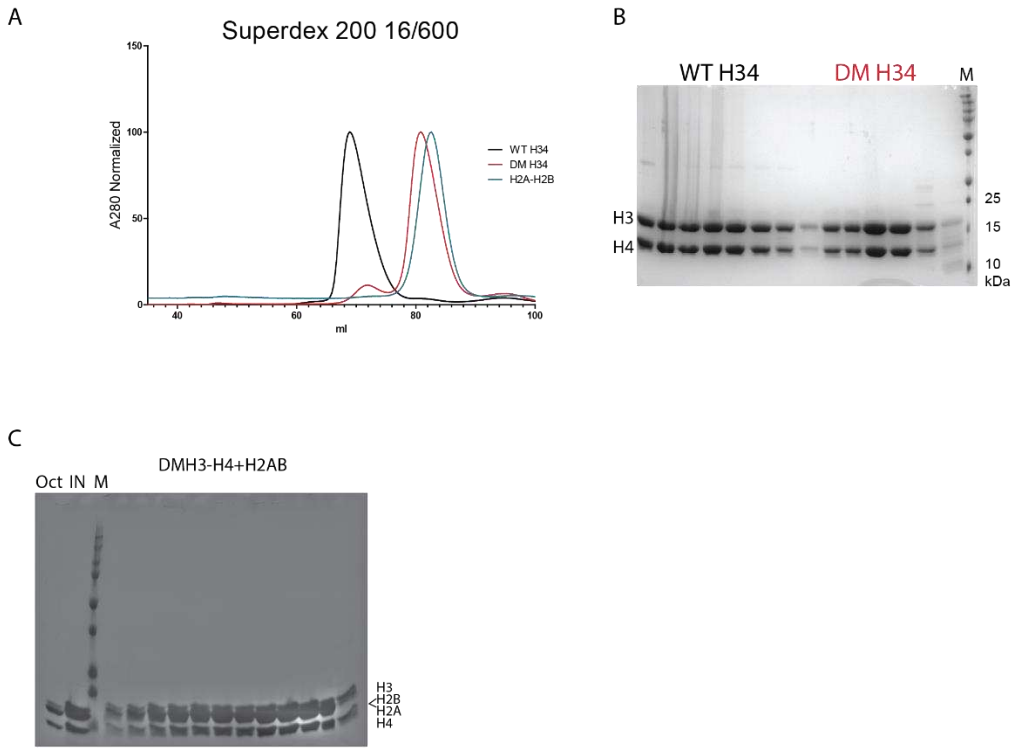


Figure 4.2: H3-H3' interface is important for proper histone octamer assembly (continued)

A.) Gel filtration chromatography of refolded histone complexes using 16/600 Superdex 200 column. The DMH3-H4+H2A-H2B peak samples are analyzed on SDS-PAGE shown in Figure 4.2 C. The A280 absorbance of each sample is normalized for comparison. B.) WT and DM H3-H4 gel filtration peak samples are analyzed by SDS-PAGE. C.) SDS-PAGE analysis of the DM H3-H4+ H2A-H2B peak samples shown in Figure 4.1 A. Oct is the purified histone octamer to show the stoichiometry of histones. IN is the loading samples before gel filtration.

step for nucleosome assembly. In Chapter 3, I have shown that the tetramerization of H3-H4 is required for chaperone mediated tetrasome assembly. Moreover, previous studies using a H3-H4 mutant that abolished the tetramerization interface affects replication-coupled nucleosome assembly and epigenetic regulation in *C. elegans* (Nakano et al., 2011), indicating this tetramerization interface is important *in vivo* for proper nucleosome assembly. Here, we want to understand what leads to these defects. We applied either WT H3-H4 or DM H3-H4, using 147 bp DNA as the substrate for tetrasome reconstitution, a similar protocol of nucleosome reconstitution with salt dilution (Muthurajan et al., 2016). Strikingly, DM H3-H4 fails to assemble into tetrasomes, but mainly forms a species containing a single H3-H4 dimer on DNA as analyzed on native PAGE (Figure 4.3), similar to what was observed with CAF-1 . While it is possible that transient tetrasomes are assembled in both processes, they could not survive during electrophoresis. The fact that neither histone chaperone nor salt reconstitution could assemble stable tetrasomes with DMH3-H4 suggests that the tetramerization interface is essential for stable tetrasome. Actually, WT tetrasome is very stable as tested before (Taguchi et al., 2014). Previous biochemical studies indicate that H3-H4 are cooperatively associated with DNA in high salt (Burton et al., 1978), and based on the nucleosome structure (which was not known at that time), we could speculate that the H3-H3' four helix bundle formation is the basis for this cooperative behavior (Luger et al., 1997). Such a cooperative mechanism could potentially explain the importance of tetramerization for stable tetrasome formation.

4.2.3 Distinct mechanisms governing nucleosome assembly by chaperone and by salt reconstitution

We have shown the importance of the H3-H4 tetramerization interface in histone octamer assembly as well as in tetrasome assembly. We next asked whether this interface is essential for nucleosome assembly. First, we attempted the salt reconstitution protocol with both WT H3-H4 and DM H3-H4, in the presence of H2A-H2B dimers. Both samples could form nucleosomes

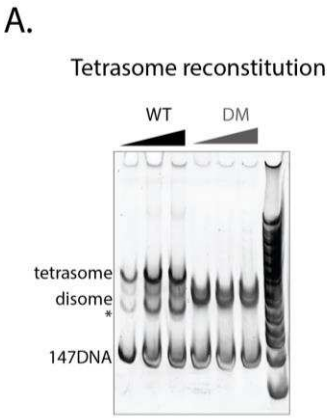


Figure 4.3 H3-H3' interface is important for proper tetrasome formation

A.) Tetrasome reconstitution by step salt dilution, analyzed on native-PAGE. 147 bp 601 sequence DNA is used at 3 μM in initial setup, and either WT H3-H4 or DM H3-H4 was titrated into DNA in 1, 1.2, or 1.4 fold excess over DNA, as indicated. The disome from DM sample runs slightly faster on native gel might be due to the introduced glutamic acid mutation.

without distinguishable difference, as analyzed through native PAGE (Figure 4.4A). DM H3-H4 could be reconstituted into nucleosomes onto both the 147 bp and 207 bp 601-derivative DNA . To further confirm the homogenous band formed on the gel is a nucleosome, we performed MNase digestion with a time course and quantified the protected fragments that indicate fully assembled nucleosome species (Figure 4.4B) (Mattiroli et al., 2017b). DM H3-H4 reconstituted nucleosome is resistant to MNase digestion, and has similar MNase resistance to the nucleosomes assembled with WT histones. This suggests that the DNA is well packaged in the DMH3-H4 nucleosome and that DMH3-H4 nucleosomes might have a structure similar to WT nucleosomes'. However, we have recently shown that the histone chaperone CAF-1 mediated nucleosome assembly is dramatically impaired when using DM H3-H4 as substrates (Mattiroli et al., 2017b), arguing that a stable $(H3\text{-}H4)_2$ tetramer is a prerequisite for chaperone-mediated nucleosome assembly. Moreover, when we assayed the histone chaperone Nap1, a well-studied histone chaperone involved in *in vitro* nucleosome assembly (Andrews et al., 2010; Fyodorov and Kadonaga, 2003), using the DM H3-H4 in the same nucleosome assembly assay setup, we did not observe nucleosome assembly compared to the control (Figure 4.4C). To quantify the nucleosome assembly activity of different histone chaperones with DM H3-H4, we applied the NAQ assay as described before (Mattiroli et al., 2017b), with normalized controls (no-chaperone reaction) (Figure 4.5 B). We found that DM H3-H4 dramatically inhibits the nucleosome by all histone chaperones tested (Figure 4.5), indicating a stable tetrasome is important for chaperone-mediated nucleosome assembly. Multiple studies have shown that tetrasome assembly is highly correlated to DNA synthesis in the wake of DNA replication, while H2A-H2B is incorporated onto the assembled tetrasome afterward (Almouzni et al., 1990; Gruss et al., 1993; Smith and Stillman, 1991b), suggesting a two-step nucleosome assembly in cells. *In vivo*, the tetramerization interface is also important for replication-coupled nucleosome assembly and epigenetic regulations (Nakano et al., 2011). Since DM H3-H4 could not form a stable tetrasome, de novo chaperone-mediated nucleosome could not be accomplished without

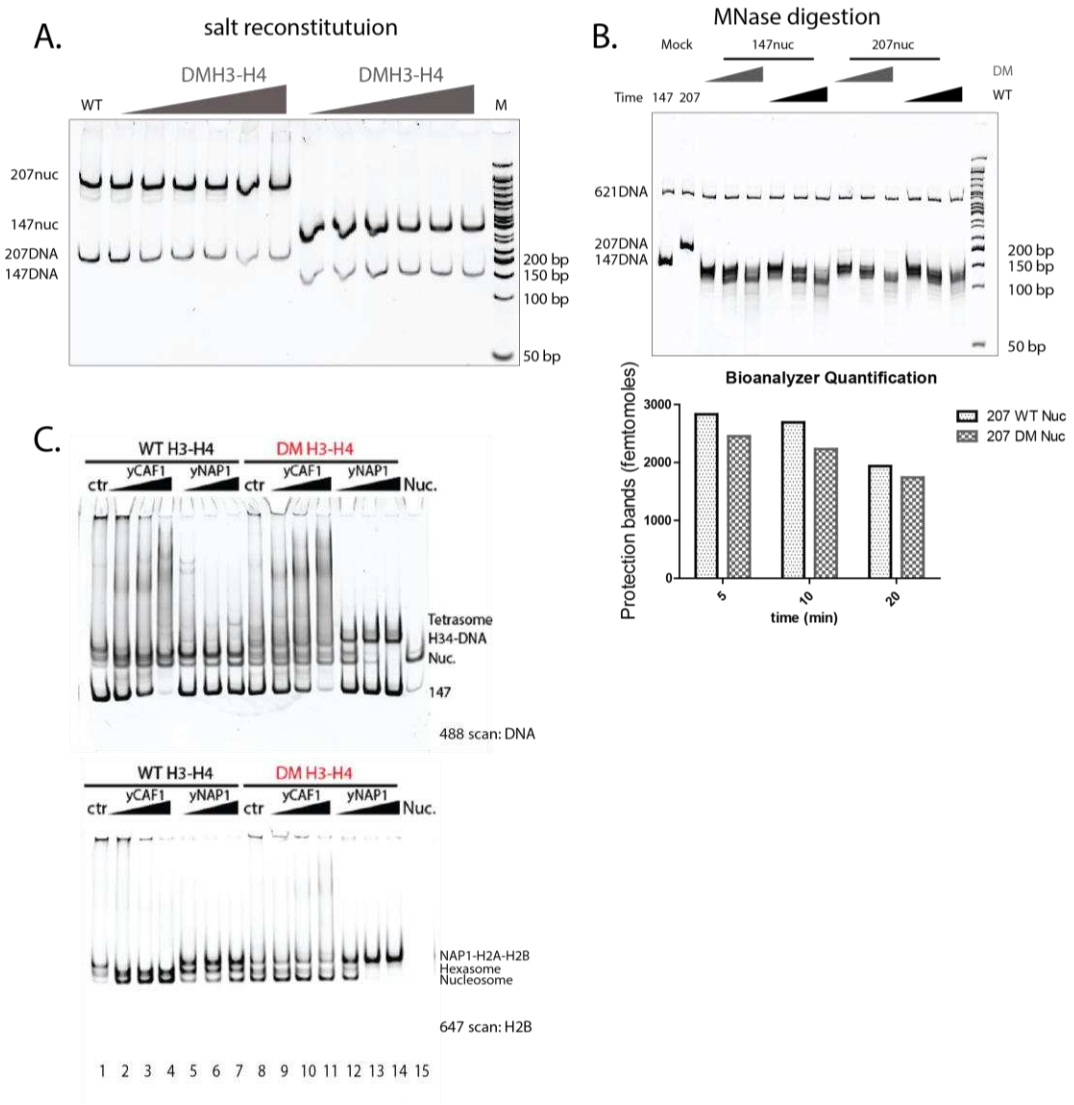


Figure 4.4 DM H3-H4 could be reconstituted into nucleosome

A.) DM H3-H4 nucleosome reconstitution with step salt dilution, analyzed by native-PAGE. Both 147 and 207 601 sequence derivative DNA are used. WT H3-H4 207 nucleosome is loaded as a control to show the position of nucleosome. Both histone H2A-H2B and DM H3-H4 are titrated to obtain optimal nucleosome assembly efficiency. B.) Time course of MNase resistance. The protected DNA bands are analyzed by native-PAGE (upper panel), and quantified by the Tapestation machine (Agilent) for nucleosomal DNA (130 bp-165 bp) (lower panel). Mock samples are treated with MNase inhibitors EDTA before adding MNase. 621 DNA is used as loading control for comparison. C.) Chaperone-mediated nucleosome assembly, analyzed by native-PAGE. 601 147 bp DNA is kept constant 100 nM, and histone H2A-H2B dimer is at 200 nM, H3-H4 heterodimer is at 200 nM for both WT H3-H4 and DM H3-H4. WT yCAF-1 is titrated at 5, 2.5 and 1.25 fold molar excess over DNA concentration, WT yeast NAP-1 is titrated at 50, 25, 12.5 fold over DNA concentration. 'Ctr' samples are the same reaction but without histone chaperone. WT nucleosome is loaded as a control to demonstrate the nucleosome position on gel.

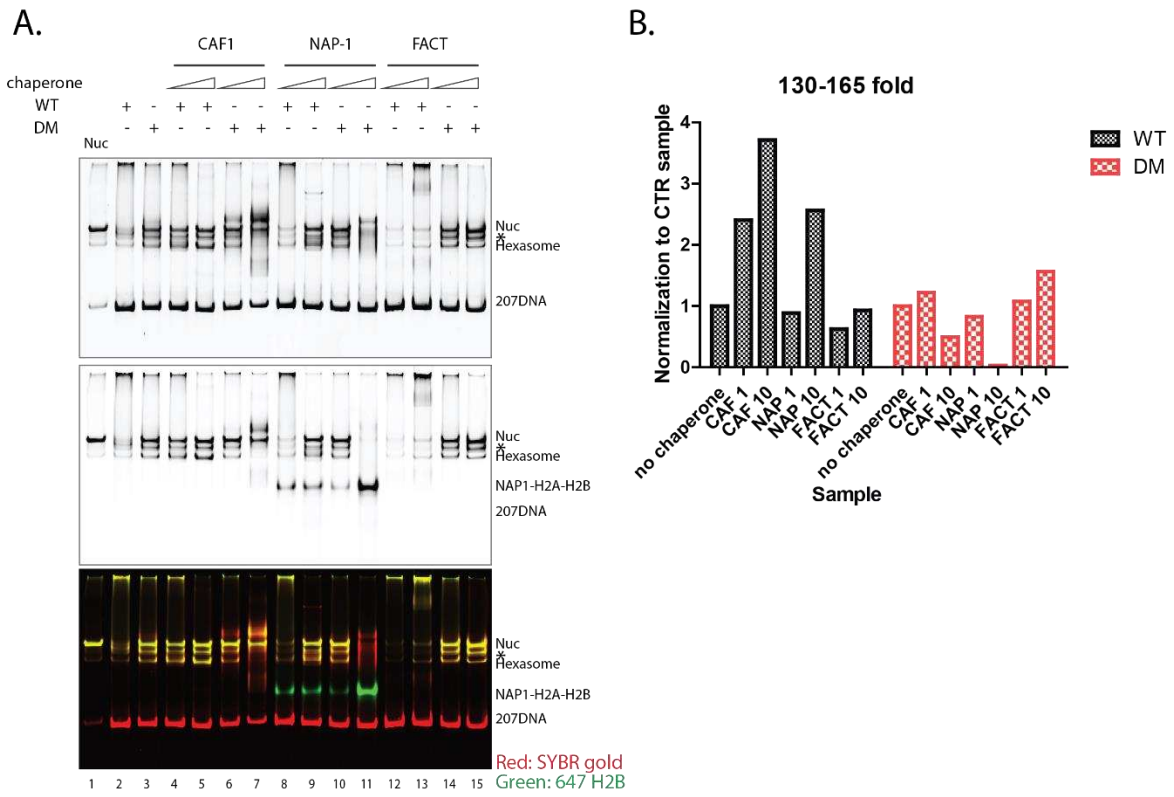


Figure 4.5 DM H3-H4 affects proper nucleosome assembly by histone chaperones.

A.) Nucleosome assembly and quantification assay (NAQ assay) analyzed by 6% native-PAGE. 601 207 bp DNA is constant at 200 nM, and histone H2A-H2B dimer is at 400 nM, H3-H4 heterodimer is at 400 nM for both WT H3-H4 and DM H3-H4. Histone chaperones yCAF1, yNAP-1 and hFACT are titrated at 10 or 1-fold molar excess over DNA. WT 207 nucleosome is loaded as a standard to indicate nucleosome position. The asterisk indicates an uncharacterized species. B.) NAQ assay analyzed by Tapestation machine (Agilent). WT H3-H4 nucleosome assembly activity is normalized to the WT H3-H4 control level, which does not contain histone chaperone. DM H3-H4 nucleosome assembly activity is normalized to the DM H3-H4 control level. (This result should be considered as preliminary as further replicates are required)

a stable tetrasome *in vitro*. Overall, our findings indicate that nucleosome assembly by histone chaperones are distinctive from salt reconstitution, which bypass the necessity of a stable $(H3\text{-}H4)_2$ tetramer.

4.2.4 H2A-H2B supports nucleosome stability by bridging two H3-H4 dimers within nucleosome

The distinctive nucleosome assembly mechanism between histone chaperones and salt reconstitution is striking as this indicates that the salt reconstitution is a binding equilibrium between histones and DNA. Moreover, the tetramerization interface of H3-H4 is dispensable for nucleosome reconstitution by salt dilution, but required for a stable histone octamer assembly in the absence of DNA, indicating DNA is involved in wrapping the "unstable" octamer in position. Histones bind to DNA with different affinities based on the DNA sequence *in vitro*, regardless of multiple regulators in the cells that localize nucleosomes along certain genome locus (Struhl and Segal, 2013). Thus, DNA would potentially change the stability of nucleosome. To test if the DM H3-H4 nucleosome could be stabilized by DNA, we used a 5S DNA, which is over 100 times weaker than the 601 sequence for nucleosome reconstitution (Lowary and Widom, 1998). Strikingly, with 5S DM H3-H4 could still be reconstituted into nucleosome (Figure 4.6A, lane 7), indicating that even lower-affinity DNA sequences are capable of stabilizing nucleosomes assembled with DM H3-H4. From the nucleosome structure, we know that each H2A-H2B can bind to two H3-H4 moieties within the nucleosome through the H2B-H4 four helix bundle and the interaction between H2A and its docking domain formed by another H3-H4 dimer (Figure 4.7 A), so each H2A-H2B dimer can potentially be involved in stabilizing two DM H3-H4 moieties within the nucleosome (Figure 4.7 A). To test this hypothesis, we introduced a mutation into H4 (H4Y98H) which blocks H2A binding to its docking site (Hsieh et al., 2013). As published, the single mutation does not disable nucleosome assembly using either 601 or 5S DNA sequence (Figure 4.6A, lane 2 and 6), but it might cause a more open nucleosome due to the loose H2A

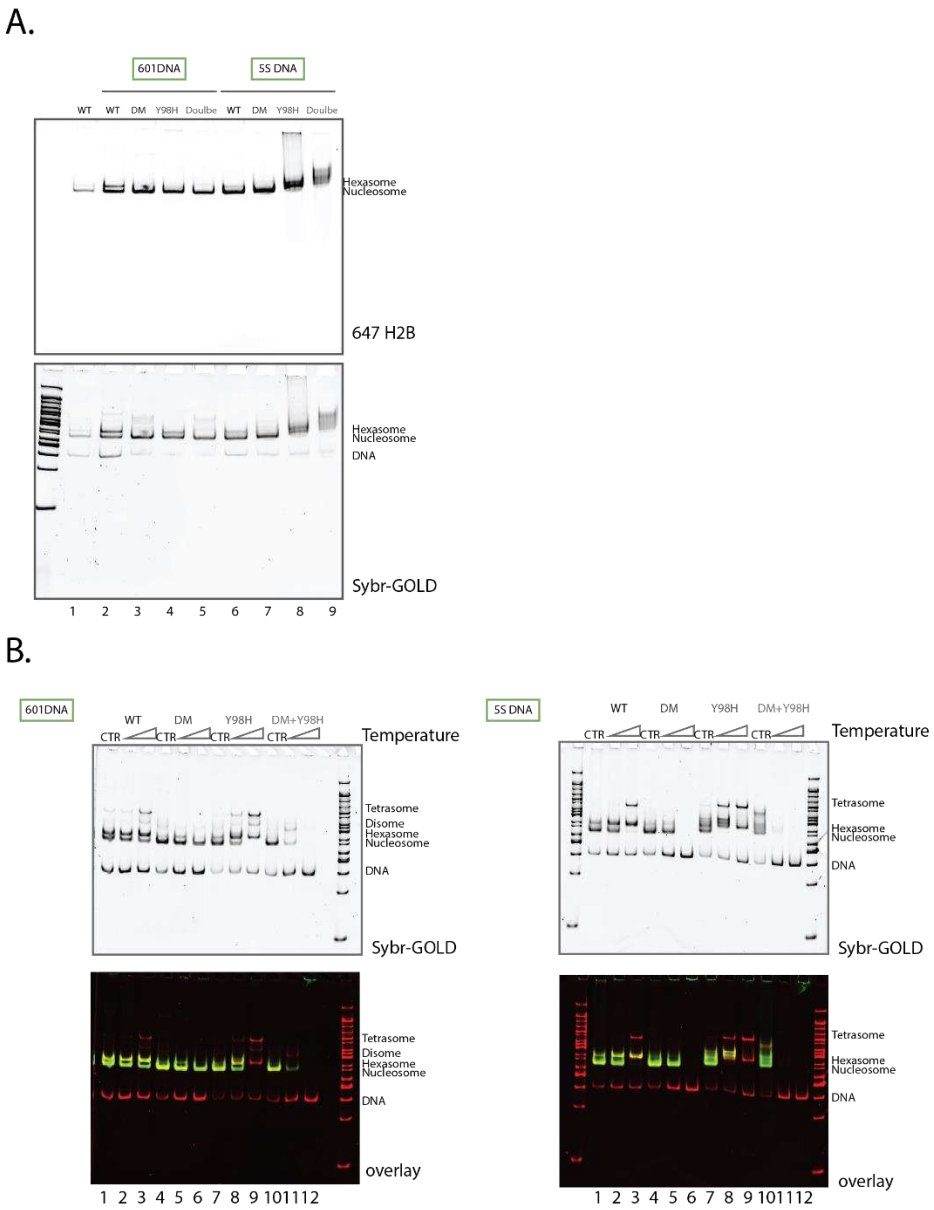


Figure 4.6 Proper H2A docking onto H3-H4 dimer is important to bridging two H3-H4 dimers within the nucleosome

A.) Nucleosome reconstitution by step salt dilution, analyzed by 6% native-PAGE. WT H3-H4 601 nucleosome is loaded as a standard to demonstrate the nucleosome position. The initial concentration of DNA for step dilution nucleosome reconstitution is 3 μM, the H3-H4 and H2A-H2B dimers are at 3.7 μM and 4 μM, respectively (both WT and DM H3-H4 concentrations are calculated as heterodimers). H2B is labeled with Atto-647N. DNA is stained with Sybr-Gold for visualization. “double” mutant species depicts combined mutation of H4 Y98H and DM H3. B .) Nucleosome stability assay by heat treatment analyzed by native-PAGE. Samples are incubated in the PCR machine for 3 hours at two different temperatures (50°C and 60°C). Ctr samples are the same samples but left on ice. H2B is labeled with Atto-647N. DNA is stained with Sybr-Gold for visualization.

A.

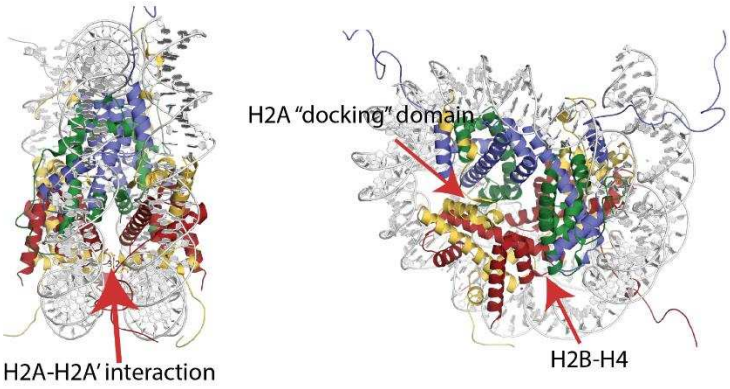


Figure 4.7 Nucleosome side view and tilted view (PDB: 1kx5).

A.) H2A is yellow, H2B is red, H3 is blue and H4 is green. The left panel shows the L1-L1' interaction between two H2A within the nucleosome, the right panel shows the two tethering sites on two moieties of H3-H4 dimers by a single H2A-H2B dimer.

adhesion when using 5S DNA, as shown by the slightly higher migration of the particles on a native gel (lane 8). Surprisingly, H4Y98H could still be assembled into nucleosomes when paired with DM H3, using 601 DNA sequence (lane 5). In contrast, when using 5S DNA, nucleosome reconstitution was dramatically impaired when both tetramerization of H3-H4 and H2A docking site are blocked (lane 9). These data suggest that DNA binding by histones can compensate for decreased histone octamer stability.

Since all histone mutants could be reconstituted into nucleosomes, we wanted to see if these proposed interfaces are important for the stability of the nucleosome. Nucleosome stability can be measured by heat treatment (Taguchi et al., 2014), during which the histone-DNA interaction would be fragile. We incubated the nucleosomes at 50 or 60 degrees for 3 hours in a PCR machine to prevent solvent evaporation. Mutations in either the H3-H4 tetramerization interface (DM H3-H4) or the H2A docking site (H4Y98H) does not change 601 nucleosome stability under heat treatment too much, but they form distinctive products (Figure 4.6B, left). On the DM H3-H4 nucleosome, when H2A-H2B is depleted, no tetrasome intermediate is observed and we could see released free DNA (lane 5 and 6), in line with the above observation that the tetramerization interface is important for stable tetrasome. However, the Y98H mutation should more easily lose H2A-H2B dimer during the heat shock, and thus the majority of the end product is tetrasome (lane 8 and 9). Strikingly, when both mutations are combined in the same nucleosome, it becomes very unstable during heat shock and no histone-DNA complexes survived after 60 degree heat shock, suggesting that the H2A-H2B dimer is involved in stabilizing two DM H3-H4 in the DM nucleosome. These data suggest that the histone interactions within the nucleosome are important for its inherent stability.

Next, we wanted to understand how DNA affinity affects the stability of nucleosomes, so we performed the same heat shock experiments with the inherently less stable 5S DNA reconstituted nucleosomes. At 60 degrees, WT nucleosomes are dissociated and form stable

tetrasomes and/or hexasomes, without releasing significant amounts of free DNA (Figure 4.6b lane3). However, DM nucleosomes with 5S DNA are totally dissociated without any histone-DNA complex left (Figure 4.6 b, right, lane 6) compared to the DM nucleosome with 601 DNA (Figure 4.6 b, left, lane 6). Consistent with the above observation, the H2A docking site mutation (H4Y98H) only affects the stability of H2A-H2B on the nucleosome, while the tetrasome could be largely sustained even after 60 degree heat treatment. However, H2A-H2B is lost more readily during heat shock when using 5S DNA compared to 601 DNA (Figure 4.6 b, lane3, lane 6 and lane 9 on both gels). Together, these data show that strong DNA-histone affinity of the 601 sequence could rule out the requirements of stable octamer assembly. Nevertheless, the interactions between histone dimer pairs are important for the stability of the nucleosome. Practically, in cells, 5S DNA is one of the stronger nucleosome positioning elements in the genome, therefore H2A-H2B is likely important for the nucleosome stability by tethering two H3-H4 moieties *in vivo*.

4.3 Discussion

Overall, we used site-directed mutagenesis and two different types of DNA to study the dynamics of nucleosome assembly and the stability of the nucleosome by heat shock. Our data showed that salt reconstitution mediated nucleosome formation is distinct from histone-chaperone mediated nucleosome assembly, for which a stable tetrasome is a prerequisite, consistent with previous observations in replication-coupled nucleosome assembly, the most massive nucleosome assembly during the cell cycles (Almouzni et al., 1990; Nakano et al., 2011; Smith and Stillman, 1991b). We found that strong DNA-histone affinity could force nucleosome assembly during step salt dilution, confirming that the 1.7 turns of DNA wrapping, a unique feature of the nucleosome is important for the stability of the nucleosome. Moreover, we provide direct evidence showing that the histone contacts within the nucleosome are important

for the inherent stability, potentially by decreasing the dynamics of histone dimer pairs within nucleosome.

We showed that, as expected, the tetramerization interface of H3-H4 is important for stable tetrasome formation. Tetrasome assembly marks the initial step for nucleosome assembly, and it is highly coupled to DNA synthesis *in vivo* (Smith and Stillman, 1991b). The stable tetrasome makes it an ideal intermediate for the later H2A-H2B incorporation during DNA replication. Moreover, we found that H2A-H2B incorporation is favored when tetrasome rather than disome is present, as more H2A-H2B dimers are trapped by the NAP-1 chaperone if tetrasomes are not properly assembled (Figure 4.5a, lane 9 and 11). Therefore, the strong four helix bundle between H3 and H3' is a crucial property for the stepwise nucleosome assembly in cells. Interestingly, both H3 and H4 have longer N terminal tails than H2A and H2B, which are subjective to various post translational modifications that act as epigenetic markers to regulate gene expression on the chromatin basis. Therefore, this highly stable tetrasome might serve as a carrier for epigenetic information during replication or transcription, which requires local chromatin dynamics (Gruss et al., 1993; Kireeva et al., 2002).

Histones are amongst the most conserved proteins in evolution. All four histones in the nucleosome share a conserved histone fold domain, which has three α helixes linked with two flexible loops. Histones are exclusively paired either in H2A-H2B or H3-H4 heterodimers, forming a stable "shake-hand" structure (Arents et al., 1991). While two H3-H4 heterodimers could tetramerize through the four helix bundle between H3-H3', H2A-H2B and H3-H4 form a weaker four helix bundle between H2B-H4 (Eickbush and Moudrianakis, 1978; Luger et al., 1997). This structurally similar but biophysically different interaction between H2B-H4 and H3-H3' might suggest an evolutionary divergence that this weak interaction of H2B-H4 is required for proper chromatin dynamics under certain situations, which are imposed by a group of factors, such as histone chaperones, chromatin remodelers and histone variants. Nevertheless,

we provided evidence showing that H2A-H2B dimers could promote nucleosome stability by tethering two H3-H4 moieties within the nucleosome through H2B-H4 and H2A-H2A docking domain interaction. We showed that this promotion is facilitated by H2A-H2B-DNA binding. This finding suggests a potential regulatory target, the H2A-H2B dimers for nucleosome disassembly, in accordance with previous observation that the H2A-H2B dissociates first during nucleosome disassembly (Gansen et al., 2009). Our study suggests two potential regulatory mechanisms toward the H2A-H2B release. One is at the H2A docking site. Multiple trans-acting factors use this site to regulate the nucleosome dynamics. For example, both chromatin remodeler Swr1 and histone chaperone ANP32E binds to H2A.Z-H2B dimers in a way that block its interaction with the docking domain (Hong et al., 2014; Obri et al., 2014), while Swr1 is involved in H2A.Z incorporation into the nucleosome and ANP32E is involved in removing H2A.Z from nucleosomes. Another regulatory site is at the H2A-H2B-DNA binding interface. Most H2A-H2B histone chaperones compete histones with DNA binding, and this feature has been applied to regulate nucleosome dynamics (Andrews et al., 2010; D'Arcy et al., 2013; Kemble et al., 2015). Alternatively, both strategies might be used for synergistic regulation of H2A-H2B release. For example, the histone chaperone FACT, which is important for maintaining nucleosomes after transcription, binds to the H2A docking domain that displaces H2A tails as well as H2A-H2B in a way that prevents DNA binding (Kemble et al., 2015; Tsunaka et al., 2016). This might facilitate Pol II progress by preventing the association of DNA back onto histones. Moreover, we know that after H2A-H2B depletion, the H3-H4-DNA complex (tetrasome) would face a challenge as Pol II goes through, which would transiently disrupt H3-H4-DNA contacts, leaving the $(H3\text{-}H4)_2$ tetramers vulnerable to the nuclear conditions as well as to chaperone Asf1. Therefore, FACT might serve as a substitution of H2A-H2B in the role of tethering two H3-H4 heterodimers during Pol II progress (Tsunaka et al., 2016). Future studies on these two regulatory mechanisms would advance our understanding of chromatin dynamic regulations during DNA-related processes.

4.4 Materials and methods

4.4.1 Cloning and reagents

cDNA for the yeast CAF-1 complex were received from Paul Kaufman. These were cloned into the MultiBac vector for expression in insect cells. Cac1 was cloned into pACEBac1, Cac3 in pIDC, Cac2 in pIDS and these were recombined by Cre-Lox as described (Bieniossek et al., 2008). A His-tag was inserted at the C-terminal end of Cac2 for purification purposes. All complexes are prepared with a short C-terminal deletion of Cac2 (1-449), predicted to be disordered. Bacmids for expression in SF21 cells were prepared as previously described (Bieniossek et al., 2008). The DNA used in the assembly and EMSA assays were based either on the 601 Widom sequence or 5S DNA sequence and were prepared as previously described (Dyer et al., 2004). Purified yeast NAP-1 is a courtesy from Hataichanok (Mam) Scherman. Purified human FACT is a courtesy from Yang Liu. DMH3 is made by introducing the mutations on the WT H3 on plasmid pET3d. The expression of mutant DMH3 is performed similarly to the one of WT H3 (Dyer et al., 2004).

4.4.2 Protein preparations

Xenopus laevis histones were purified from E.coli cells as previously described (Dyer et al., 2004), and stored in 2 M NaCl at -80°C. Labeling of histone proteins was performed as previously described (Muthurajan et al., 2016). Specifically, we labeled H2B with AttoN-647 on T112C prior refolding with the histone partner.

CAF-1 was expressed in Sf21 cells and purified using a HisTrap column (GE) in buffer containing 50 mM TRIS 8.0, 600 mM NaCl, 5% glycerol, 10 mM imidazole, 5 mM BME (beta-mercaptoethanol), in presence of COMPLETE EDTA-free protease inhibitor (Roche), DNase I, 3 mM CaCl2 and 3mM MgCl2. The complex was then loaded on a MonoQ column in buffer A (50 mM TRIS 8.0, 200 mM NaCl, 1 mM EDTA, 1 mM TCEP) and eluted with buffer B containing 1M NaCl. The protein was then injected into a size exclusion column (Superdex 200) in 30 mM Tris

pH 7.5, 300 mM NaCl, 1 mM EDTA, 1 mM TCEP. Proteins were concentrated to 1-20 mg/ml and stored at -80°C in gel-filtration buffer.

4.4.3 Nucleosome reconstitution

Nucleosome reconstitution is performed in a step-wise salt dilution method as described (Muthurajan et al., 2016). Basically, histones H3-H4 and H2A-H2B dimers are mixed, with 1.1 ratio of H2A-H2B dimer over H3-H4 dimer (considered as dimer concentration). This mixture is titrated into DNA at 2 M NaCl, with a ratio of histone octamer (considered as $(H3\text{-}H4)_2$ tetramer concentration) to DNA ranging from 1.2-1.8. This mixture volume is brought up to 10 µl with DNA concentration set at 3 µM. Then 5.2 µl, 6 µl, 14 µl and 32 µl of dilution buffer (20 mM Tris-HCl pH7.5, 1 mM EDTA, 1 mM BME) is added in a stepwise, and each step include 30 min incubation at 37°C. Finally, add 67.2 µl of dilution buffer to bring down salt to ~200 mM.

4.4.4 Tetrasome reconstitution

Tetrasome reconstitution is done in a similar manner as nucleosome reconstitution. However, the H2A-H2B dimers is omitted from the experiment setup.

4.4.5 Time course MNase digestion

Reconstituted nucleosomes are diluted to ~ 200 nM. The samples were then diluted to a DNA concentration of 50 nM in 200 µl digestion reactions. 50U of MNase enzyme was added in a final buffer containing 50 mM TRIS pH 7.9, 5 mM $CaCl_2$. After incubation at 37°C for 5 min and 15 min, at each time point, take 100 µl mixture out and quench with 10 µl of 500 mM EDTA, pH 8. The mock nucleosome samples are treated with EDTA before MNase enzyme was added into the mixture. The quantification of protected DNA (nucleosomal DNA) by bioanalyzer (Agilent) is as described in NAQ assay (see chapter 2 and 3).

4.4.6 Nucleosome assembly assay

This nucleosome assembly assay was carried out in buffer (25 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.02% Tween-20). 147 bp 601 DNA is kept constant 100 nM, and histone H2A-H2B dimer is at 200 nM, H3-H4 heterodimer is at 200 nM for both WT H3-H4 and DM H3-H4. WT yCAF-1 is titrated at 5, 2.5 and 1.25 fold molar excess over DNA concentration, WT yNAP-1 is titrated at 50, 25, 12.5 fold over DNA concentration. Histones are first mixed with chaperones and incubated for 10 min at room temperature, and then DNA is added and incubate another 30 min. The products of the assembly reaction are resolved on 6% native PAGE run for 1 hour at 150 V in cold cabinet. Gels were stained with SybrGOLD for DNA and imaged both 488 and 647 channels (H2B) on a Typhoon FLA 9500 (GE).

4.4.7 NAQ assay

The assembly assay was carried out as described above containing 200 nM of 207 bp DNA, 200 nM $(H3\text{-}H4)_2$, 400 nM H2A-H2B and titration of CAF-1 or other histone chaperones (200 and 2000 nM). After the assembly reaction, the samples were diluted to a DNA concentration of 50 nM in 100 µl digestion reactions. 25U of MNase enzyme was added in a final buffer containing 50 mM TRIS pH 7.9, 5 mM $CaCl_2$. After incubation at 37°C for 10 minutes, the reactions were quenched with 10 µl of 500 mM EDTA, pH 8. The DNA was then purified using a modified protocol of the MinElute kit from QIAGEN. 550 µl of PB buffer and 10 ul of 3M sodium acetate were added to each sample and they were incubated at room temperature for 10 minutes. At this point, 50 ng of DNA loading control (or reference band, a 621 bp DNA fragment) was added to each tube. The samples were applied to the MinElute spin column and washed as prescribed by QIAGEN. The DNA was eluted with 10 µl of water. 1 µL of the eluate was used to load a DNA 1000 chip on the Bioanalyzer machine (Agilent), and 2.5 µl were loaded on a 10% PAGE gel. The gel was run for 45 minutes at 200 V in 0.5x TBE buffer at room temperature. Gels were stained with SybrGOLD for DNA and imaged on a Typhoon FLA 9500 (GE). The

Bioanalyzer data was analyzed using the Agilent Expert 2100 software. The signal threshold was set at 20 RFU. The reference band was corrected for the proper size (621 bp) and the calculated molarity values were used to normalize all other bands present in the lane. These normalized values were then normalized to the Control sample (WT histone plus DNA, but no chaperones) for comparison. Nucleosome signal was calculated from protected bands ranging between 126-160 bp in length, based on the digestion of salt-reconstituted nucleosomes.

4.4.8 Nucleosome stability assay by heat treatment

Reconstituted nucleosomes are diluted to ~ 80 nM (NaCl concentration is ~60 mM). Nucleosome are split into 10 µl aliquots. Prepare each type of nucleosome with 3 aliquots. One is incubated on ice as a control, the other two are incubated in the PCR machine for 3 hours at two different temperatures (50°C and 60°C, respectively). At the end of reaction, 2 µl of 50% glycerol was added into the nucleosome sample. Load 6 µl of the mixture on 6% native gel, and run at 150 V for 60 min at 4°C. Gels were stained with SybrGOLD for DNA and imaged both 488 and 647 channels (H2B) on a Typhoon FLA 9500 (GE).

CHAPTER 5: SUMMARY AND PERSPECTIVE

This dissertation demonstrates the defined regions involved in yeast CAF-1 subunits assembly, as well as the histone binding modules on yeast CAF-1 (chapter 2). Moreover, by using a new quantitative method (NAQ assay) to measure the nucleosome assembly activity by histone chaperones, we demonstrated the direct histone deposition mechanism by CAF-1 (or any histone chaperone) (chapter 3). This nucleosome assembly mechanism by CAF-1 allows me to revisit the importance of H3-H4 tetramerization for the nucleosome assembly. And finally I found that the nucleosome assembly mechanism is different between salt-driven process and chaperone-mediated process, which requires proper tetramer assembly (chapter 4).

We demonstrated that CAF-1 has an elongated shape, and that the large subunit is the platform for arranging another two WD40 repeat containing subunits. The histone binding core is attributed to the ED-rich region and Cac2, the middle subunit, while the WHD domain, which binds to DNA, plays important roles for coordinating two histone-bound CAF-1 on DNA for proper tetrasome assembly. We showed that CAF-1 binds to the lateral surface of H3-H4 thus competes with DNA binding, and only accommodates one H3-H4 heterodimer. This binding mode requires two CAF-1 for synergistic tetrasome assembly, suggesting that histone H3-H4 is handled as the dimer form all the way right before its incorporation onto DNA.

This nucleosome assembly mechanism by CAF-1 proposed here suggests a potential semi-conservative H3-H4 segregation pathway, however, previous studies suggested that parental histone H3.1 is not mixed up with newly synthesized histone H3.1 (Xu et al., 2010). Thus, how CAF-1 coordinates different pools of histones for proper H3-H4 segregation remains a challenge yet to be investigated. One possibility is that CAF-1 is only involved in deposition of new

histones, or that proper regulation exists for CAF-1 to allow it to distinguish between new and old histones. It is likely that CAF-1 is only involved in deposition of new histones, as supported by *in vivo* immunoprecipitation (Verreault et al., 1996), thus alternative pathways might exist for parental histone recycling. For example, the histone chaperone FACT has been shown to be involved in the disassembly of the nucleosome ahead of the replication fork, in cooperation with the MCM helicase complex (Tan et al., 2006). Recent structural studies also suggest the existence of a ternary complex between MCM2, $(H3\text{-}H4)_2$ tetramer and FACT (Huang et al., 2015b; Tsunaka et al., 2016). FACT has been proposed to deposit recycled parental histones and new histones following the replication fork (Foltman et al., 2013; Yang et al., 2016b). As FACT binds to H3-H4 in its tetramer form that is similar to its structure within nucleosome (Tsunaka et al., 2016), this co-chaperoning mechanism might suggest a conservative segregation of parental histone H3-H4 after replication. Another possibility is that CAF-1 delivers both old and new histones, but in this case specific regulations are required from CAF-1 as both type of histones are escorted by Asf-1 (Groth et al., 2007a), which handles H3-H4 dimer rather than a tetramer. For example, there might be two populations of CAF-1, one of which might be involved in close association with chromatin for recycling old histones, while the other one would be free in the nucleus to accept new histones. This is supported by the finding that nearly half of CAF-1 is tightly associated with chromatin independent of PCNA (Hoek et al., 2011), and that the KER region might be responsible for such CAF-1 dwelling (Sauer et al., 2017). Moreover, a combination of different p60 phosphorylation states starts to emerge when cells enter into S phase (Marheineke and Krude, 1998), supporting the existence of diverse populations of CAF-1 for specific functions such as distinguishing new and old histones. Future studies on these questions would boost our understanding of the proper histone segregation, which is the basis for subsequent propagation of epigenetic markers. Alternatively, there is a third possibility that the conservative segregation of H3-H4 stems from chaperones that function upstream of CAF-1, perhaps Asf1. It is intriguing to imagine that Asf1 might only handle new histones. In this case,

since CAF-1 receives histone H3-H4 from Asf1, this will ensure CAF-1 handles only new histones, so that parental histones are recycled and transferred in a different mechanism. This is possible because previous studies found that the Asf1 association with parental histones is under stressed condition where replication is stalled (Groth et al., 2007a; Jasencakova et al., 2010). This will hamper the proper recycling and transfer of parental H3-H4 and end up with H3-H4 falling off chromatin and being captured by Asf1.

These studies also highlight that DNA binding is an important and potentially unique feature of histone chaperones that are dedicated to nucleosome assembly. In this study, we clearly showed that H3-H4 lost the DNA binding affinity when bound to CAF-1 (Figure 3.6d), thus proper guidance of histone onto DNA is the fundamental task falling on the shoulder of the histone chaperone. Particularly, DNA binding by CAF-1 in complex with histones is not only important for its localization to substrate DNA, but also the cooperative manner of DNA binding ensures correct pairing of two H3-H4 dimers for tetrasome assembly. Since the tetramer is assembled with the assistance of DNA, this direct DNA involvement would further facilitate the histone transfer from chaperone to DNA by tetramer formation, as the tetramerization of H3-H4 would reinforce the DNA-histone contact by a cooperative binding manner (Burton et al., 1978). Interestingly, DNA binding by the WHD is activated by histones, which is a clever mechanism to ensure efficiency of histone deposition. Not all histone chaperones contain a DNA binding motif, nor can all histone chaperones assemble nucleosomes. Therefore, by comparing histone chaperones together with their DNA binding ability would allow us to revisit histone chaperones to specifically define their functions in the future.

Indeed, there are a few histone chaperones in addition to CAF-1 with DNA binding capacity that is important for histone deposition. For example, the histone chaperone HIRA is involved in the deposition of histones in a replication-independent manner (Tagami et al., 2004). HIRA also contains a DNA binding motif, and has been shown to deposit the histone variant H3.3 in a gap-

filling mechanism (Ray-Gallet et al., 2011). The DNA binding ability of Rtt106 is important for its function in heterochromatin formation in yeast (Liu et al., 2010), although the molecular mechanism remains unknown, but it could possibly be due to the impaired histone deposition. Scm3, the yeast homologue of HJURP, is a centromeric histone chaperone important for the metabolism of CENP-A (Sanchez-Pulido et al., 2009). Scm3 also contains a DNA binding domain, which prefers the AT-rich DNA found at centromeres (Xiao et al., 2011). This DNA binding domain has been shown to be important for its histone deposition *in vitro* (Xiao et al., 2011). Therefore, it would be intriguing to test the nucleosome assembly mechanism by looking into DNA binding properties of these histone chaperones. It would be intriguing to see whether a similar dimerization mechanism might exist as a common strategy for histones chaperones that deposit $(H3\text{-}H4)_2$ tetramers onto DNA.

The fact that this study, along with recent biophysical studies which exclusively used yeast CAF-1 (Liu et al., 2016; Sauer et al., 2017), suggested that yeast CAF-1 is a bona fide candidate for future structural studies, while the conservative function of CAF-1 demonstrated thus needs further verification using its homologues in other species. However, considering the conservative function of CAF-1, the structural mapping of CAF-1 complex assembly as well as CAF-1-H3-H4 interface provides unprecedented insight for future structural studies using crystallography or cryo-EM. The proposed model here would be interesting to test in future structural studies that would demonstrate the conformational effect of these functional domains in the pairing of two CAF-1-H3-H4 complexes in the way of assembling tetramer.

Recent research from another lab also highlights that the KER region on Cac1 is important for CAF-1 DNA binding and pairing of two CAF-1 for proper tetramer deposition (Sauer et al., 2017). Unlike the WHD domain, which binds both short and long DNA, the KER region prefers to bind longer DNA than short ones (Sauer et al., 2017). The distinctive DNA binding preference for the WHD and KER region on the large subunit would be interesting for future studies to see

how these two DNA binding domains are coordinated for either CAF-1 recruitment as well as efficient tetramer deposition. Moreover, the physically separated regulatory N-terminal region and chaperoning C-terminal region might require inherent regulation to coordinate the CAF-1 recruitment, sensing cell cycles as well as efficient histone deposition. It makes sense that the middle subunit is necessary for efficient histone binding and nucleosome assembly as the middle subunit, a unique WD40 protein is exclusively functioning in CAF-1 complex *in vivo*. This might suggest a novel role for WD-40 proteins that is distinct from being an adapter or responsible for histone binding. Considering the interaction of this subunit with Asf1 and its specific hypophosporylation during S phase, more studies onto this middle subunit could reveal the structural effect of this subunit on CAF-1 since it is bound to the critical region between the ED-rich region and the WHD. For example, the middle subunit might regulate the critical WHD activation step when histone H3-H4 is engaged. On the other hand, the requirement of Cac3 for efficient heterochromatin maintenance *in vivo* and the dispensability of this subunit in nucleosome assembly *in vitro* might suggest this small subunit is regulating the epigenetic maintenance independent of nucleosome assembly. Moreover, numerous studies suggest that the WD40 proteins, including the small subunit of CAF-1, is involved in histone modifications by presenting the tails to histone modifiers (Cao and Zhang, 2004; Ruthenburg et al., 2006; Schuetz et al., 2006; Verreault et al., 1998). Therefore, it is interesting to test if the small subunit is involved in certain post-translational modifications of pre-nucleosomal histone H3-H4 (see some preliminary results in appendix I).

In summary, the semi-conservative histone deposition mechanism by CAF-1 demonstrated in this study indicates that additional regulation is required for the precise pairing of H3-H4 dimers in massive chromatin re-arrangements in the wake of replication. This could be based on other histone chaperones, specific modifications on histones, dynamic modifications on CAF-1, chromatin remodelers as well as other epigenetic regulators. Future studies on these

regulations would advance our understanding of epigenetic maintenance mechanisms during cell divisions.

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APPENDIX I: THE SMALL SUBUNIT OF CAF-1 SHIELDS H3 TAILS FROM DNA BINDING

As discussed above, the small subunit forms WD-40 β-propeller structure, which is a common protein structure seen in complexes involved in histone metabolisms (Suganuma et al., 2008). For example, the small subunit of CAF-1 is also a subunit of other protein complexes such as the remodeling complex NURF, the histone deacetylase complex NuRD and the Polycomb respressive complex 2 (PRC2) (Barak et al., 2003; Nowak et al., 2011; Zhang et al., 1999). The binding mode of WD-40 to histones has suggested that the β-barrel structure might serve as a presenter of H3-H4 for optimal post-modification by the enzyme within a complex (Ruthenburg et al., 2006; Schuetz et al., 2006; Suganuma et al., 2008). But it might also serve as a selection tool to distinguish specifically histone PTMs (Schmitges et al., 2011). Biophysical studies of p55 (the fly homologue of p48) with H3 and H4 peptides indicates that the p55 binds a single H3-H4 dimer, but in an unconventional H3-H4 conformation (Nowak et al., 2011; Song et al., 2008; Suganuma et al., 2008). In this study in chapter 2, we have shown that removal of the small subunit in yeast CAF-1 does not change the affinity of CAF-1 to H3-H4, which indicates the small subunit is not a major contributor for histone binding. However, clear evidence suggested CAF-1 is involved in epigenetic maintenance. Is CAF-1 simply a chromatin-block-building worker, without any furnishing (modification)? Does CAF-1 coordinate with other factors for epigenetic maintenance (Liu et al., 2017; Loyola et al., 2009; Quivy et al., 2008; Sarraf and Stancheva, 2004)? To answer these questions, we first need to know how CAF-1 binds to histone H3-H4, to see if CAF-1 has the potential to modify histones while on its duty.

I looked into the tails of histone H3-H4, which holds numerous positions for PTMs important for chromatin regulations (Strahl and Allis, 2000). I took advantage of various CAF-1 truncations as well as the EMSA assays. As we know from chapter 3, titration of tCAF-1_ΔWHDb-DMH3-H4

complex does not shift free DNA, as it lacks the WHD (Figure S1a, lane 5-7). However, it is noticeable that histone H3-H4 tails contain positively charged residues, which has the potential to bind DNA. As Cac3 alone could bind histones, I removed Cac3 from the CAF-1 complex, and use HBM (refer to Figure 2.6b) to replace tCAF-1_ΔWHDb in the same setup. Surprisingly, HBM-DMH3-H4 complex restores the DNA binding ability (Figure S1a, lane 8-10). This indicates that Cac3 might inhibit the histones from DNA binding. To investigate which part of the complex is involved in histone binding, I first made a short tail deletion on the basis of dimer mutant histone H3 (DMH3TL38), and refolded with WT H4, named H3TL38-DM (Figure S1b). Interestingly, when HBM is mixed with H3TL38-DM, this complex could not bind DNA anymore (Figure S1b, lane 8-10). This data suggests that the tail of H3 might contribute to DNA binding within the HBM-H3-H4 complex. Moreover, although the H4 tail contains several lysine and arginine, it seems to be inhibited from DNA binding when bound to HBM. So I made a H4 tailless mutant H4TL27, and refolded with WT H3, named H4TL27 WT (Figure S2). The H4TL27 H3-H4-HBM complex could bind to DNA, similar to H3-H4-HBM (Figure S2), indicating other parts of the complex rather than the H4 tail is involved in DNA binding in this setup. This could mean the H4 tail is shielded from DNA binding when in complex with the HBM. However, it is still unclear which part of HBM binds to the H4 tail. It is likely that the acidic region of the HBM might involve in H4 tail binding. As expected, when using a complex tCAF-1_ΔWHDb to mix with different histone mutants, none of them could bind DNA (Figure S2, lane 10-15). This data indicates that the Cac3, small subunit of yeast CAF-1, binds to the H3 tail within the complex. That means all the DNA binding interfaces on histones are shielded by CAF-1, including the histone tails and the DNA binding interfaces involved in nucleosome formation. This appendix reinforces the conclusion from chapter 3 that CAF-1 binds to the lateral surface of the H3-H4 dimer, and necessitates a DNA binding domain from CAF-1 for proper guidance of histones onto DNA. Considering the numerous PTM targets on histone tails as well as CAF1 role in epigenetic maintenance, this study would trigger the interests to probe the functions for this

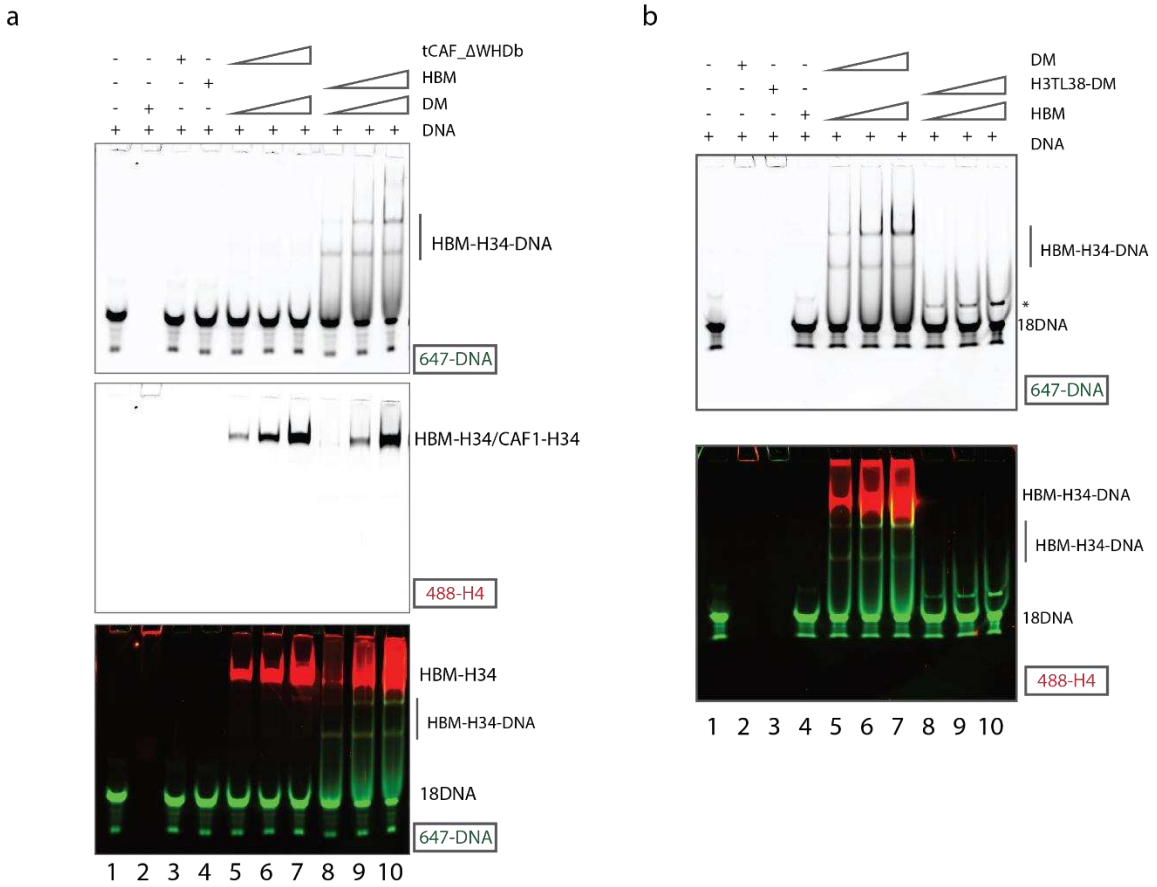

Figure S1 a.) EMSA of chaperone-histone complex to DNA. HBM or CAF-1 mutant was pre-mixed with 1.1:1 with DMH3-H4 dimers. This complex is titrated into 100 nM 18 bp DNA, with concentrations of 0.3 μM, 1 μM and 3 μM. In lane 2-4, proteins are at 1 μM. The EMSA buffer is 25 mM Tris-HCl pH 7.5, 50 mM NaCl, 1 mM EDTA, 1 mM TCEP. Histone H4 is labeled with Alexa-488, DNA is stained with SYBR-Gold. b.) EMSA of chaperone-histone complex to DNA. HBM or CAF-1 mutant was pre-mixed with 1:1.1 with H3TL38-DMH34 dimers (H3TL38 represents 1-38 aa truncated for H3). This complex is titrated into 100 nM 18 bp DNA, with concentrations of 0.3 μM, 1 μM and 3 μM. In lane 2-4, proteins are at 1 μM. The EMSA buffer is 25 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM TCEP. Histone H4 is labeled with Alexa-488, DNA is stained with SYBR-Gold.

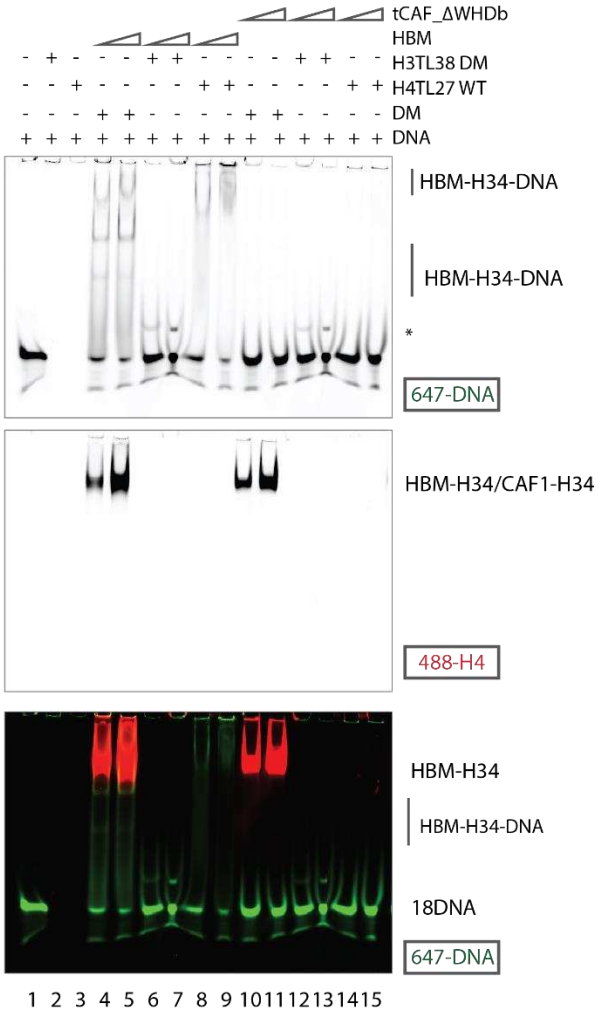

Figure S2 a.) EMSA of chaperone-histone complex to DNA. HBM or CAF-1 mutant was pre-mixed with 1:1.1 with various histone H3-H4 dimers (H3TL38 represents 1-38 aa truncated for H3; H4TL27 represents 1-27 aa truncated for H4). This complex is titrated into 100 nM 18 bp DNA, with concentrations of 1 μM and 3 μM. In lane 2 and 3, proteins are at 1 μM. The EMSA buffer is 25 mM Tris-HCl pH 7.5, 50 mM NaCl, 1 mM EDTA, 1 mM TCEP. Histone H4 is labeled with Alexa-488, DNA is stained with SYBR-Gold.

binding mode. This could potentially help answer whether CAF-1 is involved in particular histone modifications while it is building chromatin blocks, or CAF-1 has a preference for certain modified histones for proper epigenetic maintenance.