In vitro co-expression chromatin assembly and remodeling platform for plant histone variants

(植物変種ヒストンを用いた in vitro 共発現系によるクロマチン構造およびリモデリング機能再構築法の確立)

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1 CHAPTER 1: General introduction

1.1 Chromatin organization and epigenetics

The eukaryotic genome is compacted and organized into chromatin; a long-range nucleoprotein complex made up of repeating units of nucleosomes. A nucleosome consists of a histone octamer, comprising two copies each of canonical histones H2A, H2B, H3, and H4, and approximately 147 bp DNA, which wraps around the octamer in ~1.7 turns [1]. Chromatin conformation is highly dynamic, which enables adopting different structures thus functions depending on different phases in the cell-cycle in all eukaryotes. In the interphase, chromatin is present in a less compacted organization compared to the reported 30 nm chromatin fiber, whereas in metaphase, chromatin goes through condensation and adapts a highly compact conformation that forms a distinctive X-shape [2].

Chromatin structure can be divided into heterochromatin and euchromatin based on the microscopic observation of the chromosome stained by a Giemsa reagent. By definition, regions of tightly condensed chromatins are referred to as heterochromatin. Heterochromatin can be further categorized as facultative heterochromatin and constitutive heterochromatin. The former links to transcriptionally silent regions and the latter is found in the regions containing repetitive DNA sequences such as the centromeres and telomeres in chromosomes [3]. There are heterochromatin-specific proteins that are thought to physically perturb the occurrence of gene activation, DNA repair, and others. On the other hand, euchromatin regions form a looser conformation and enable a permissive state where the underlying DNA is more readily accessible for DNA-templated processes [4].

Chromatin is not solely a structural scaffold for the genomic DNA, but also a highly complex regulatory hub. Chromatin undergoes regulation at multiple levels, including post-translational modifications of both DNA and histones, formation of nucleosome core particles with alternative histone DNA stoichiometries and the incorporation of histone variants into nucleosomes [5–7]. These epigenetic regulatory processes create a complex interacting network, affecting higher-order DNA packing, and various cellular processes, including transcriptional regulation, replication, DNA repair, and others [5,8,9].

Among them, post-translational modifications are intensely studied as their presence can directly affect the stability of nucleosome-DNA interactions which correlate with permissive or repressive epigenetic states or establish signal transduction pathways for distinct cellular processes [10,11]. Chemical modifications are primarily found in the N-terminal tails of histone proteins, H2A, H2B, H3 and H4 that protrude out of the nucleosomes, offering an easily accessible surface for histone modifying enzymes to act upon as substrates. Examples of these chemical modifications include acetylation, methylation, phosphorylation, ubiquitination and SUMOylation [5].

In addition to chemical modifications, another layer of chromatin regulation is achieved through the deposition of histone variants. Histone variants convey unique properties to nucleosomes that affect chromatin stability [12], selectivity to epigenetic enzymes [13,14], and higher-order chromatin structure [15,16].

1.1.1 Histone variants in chromatin regulation

By the general definition, four core histones, including histones H2A, H2B, H3 and H4, can be divided into two categories, the replicative (canonical) histones and the replacement histones (histone variants). Canonical histones make up the majority of the nucleosomes in the eukaryotic genome. They are expressed in the DNA synthesis phase (S phase) of the cell cycle, supplying histone proteins for the formation of chromatin on the nascent genome. To accommodate the mass expression required for chromatin assembly, they are present in large intronless multicopy arrays. In contrast, histone variants are non-allelic isoforms of the canonical histones, evolved independently from canonical histones to serve specialized functions. Histone variants are encoded by one or a few genes in the genome and expressed not only in the S phase but also in other cell cycles, incorporating into the chromatin in a replication-independent manner. Histone variants retain the distinctive structural features of histone proteins, including the histone fold domain and extended N-terminal unstructured tails [7,17]. However, their amino acid sequences can differ from a few to dozens of amino acids compared to their canonical counterparts. It is also known that even a few amino acid differences can affect nucleosome properties in a profound way,

i.e., influencing stability, post-translational modifications, or recognition by histone chaperones, in turn resulting in a diverse chromatin landscape. Histone H2A and H3 variants are most studied and some of their members, including H2A.X, H2A.Z, H3.3 and the centromeric H3 (CenH3), are so-called "universal" histone variants, that are thought to be evolved in early eukaryotes. Consistently their functions are well conserved throughout nearly all species, while other histone variants evolved more recently and lineage specifically [18]. H2B variants are diverse across organisms with a limited literature, however recently H2B variants are gaining more attention and new variants are being described [19,20]. In contrast, the variants of histone H4 can only be found in a limited species, including a recently described human H4-like protein type G, which was determined as a type of H4 specifically expressed in breast cancer cells [21].

1.1.2 Histone variants in the model plant, *Arabidopsis thaliana*

Histone variants have been also described in various plants from algae to angiosperms [20,22,23]. Most of the data accumulated on histone variants in plants derive from studies featuring the model organism *Arabidopsis thaliana*. In *A. thaliana*, there are four types of histone H2As, including the canonical H2A, and three variants, H2A.X, H2A.W, and H2A.Z. Variants differ from canonical H2A by the length or unique motifs of their C-terminal tails, and conserved residues in their histone fold domains [22,24]. H2A.X is known to play a critical role in signaling DNA damage response through C-terminal phosphorylation [9,10,25]. H2A.Z is reported to be mainly associated with transcriptional regulation in plants [26,27] and other eukaryotes including humans [28] and yeast [29]. H2A.W is a flowering plant-specific histone variant, found almost exclusively in heterochromatin [15] and acts in DNA damage response in these highly condensed regions [9].

Arabidopsis H2Bs are classified into three groups based on their amino acid sequences and expression patterns [20]. Class I H2Bs, wherein H2Bs with canonical histone-like features are categorized, mainly function in somatic tissues, and show peak expression levels in actively dividing cells [20,30]. Class II/III H2Bs are known to control chromatin structure in reproductive tissues [20,31]. Class III only includes a single member, HTB8, which differs significantly from other variants and is anticipated to play a part in

chromatin condensation in sperm [31]. However, our current understanding of the physiological functions of H2B variants in *Arabidopsis* is still limited.

Three main histone H3s are known in *A. thaliana*, including the canonical H3.1 and the variants H3.3 and CENH3, all of which are known to incorporate into different regions of the genome [32,33]. The canonical H3, H3.1, is expressed in a replication dependent manner and mainly enriched in silent regions of the genome, including transposable elements, pericentromeric heterochromatin, and heterochromatic regions on the chromosome arms [7,32]. In contrast, the replacement variant H3.3, tends to be found in the regions with actively transcribed genes and harbors epigenetic modifications that link to transcriptional activation [34]. The replacement of the canonical H3.1 with H3.3 is also reported as a marker for cell fate transition from cell proliferation to differentiation [35]. The centromeric H3, CENH3, is exclusively found in the centromere of chromosomes and is reported to aid in chromosome segregation through directing kinetochore assembly [36,37]. The functions of H3.3 and centromeric H3 are well-conserved across eukaryotes. In addition to the abovementioned three H3s, a fourth group, wherein other H3-like histones or atypical histone variants are reported [38]. The function of two atypical variants have been reported in recent years, including H3.15 which facilitates wound healing through callus formation [39], and H3.10 which prevents the inheritance of the epigenetic mark H3K27me3 in sperm [40]. However, their mechanism of action and the roles of the remaining atypical H3s in chromatin functions are still largely elusive.

To note, there is no histone H4 variant in *A. thaliana*.

Plants, being sessile organisms, highly rely on environmental response mechanisms for survival and successful reproduction. Histone variants are identified as key players in stress responses. For instance, H2A.Z was reported to be modulating the so-called response genes, which are responsible for environmental and developmental cues, governing temperature sensors, plant immunity, flowering time, and growth [41]. Flowering plants have their unique ways of managing chromatin organization and epigenetic marks in their reproductive processes. Unlike mammals, flowering plants do not replace histones with protamine in their sperms to condense and reset epigenetic marks, but instead have evolved their own distinct histone variants that fulfill similar tasks. The H2B.8 histone variant facilitates condensation in the

sperm chromatin through phase separation [16], while an H3.10 replacement variant takes part in the epigenetic resetting of the repressive histone mark H3K27me3 during sperm differentiation [13,42]. A recent report on H3.3 suggested that it plays a part in post-embryonic development in seeds [43]. By understanding the functions of histone variants in molecular mechanisms related to environmental responses and reproduction, we can gain valuable insights into plant adaptation and resilience in the face of environmental challenges.

1.1.3 The role of chromatin remodelers in chromatin regulation

Nucleosomes create a structural barrier between the underlying DNA sequence and regulatory proteins; therefore, the position and the mobility of nucleosomes need to be under stringent regulation to allow access to the DNA for downstream processes. Chromatin remodelers are protein complexes that evict, assemble, or slide nucleosomes alongside the DNA through ATP hydrolysis, thereby controlling DNA accessibility. Remodelers can execute specific roles through their unique domain structures and are even involved in "nucleosome editing" or the exchange of histones to histone variants in nucleosomes [44,45]. Chromatin remodelers could be grouped into four subfamilies based on their ATPase core domains, many of which are conserved in yeast, human, and plants. These subfamilies include the SWI/SNF (switch/sucrose-non-fermenting), ISWI (imitation switch), CHD (chromodomain-helicase-DNA binding), and INO80 (inositol requiring 80) family remodelers [44].

Remodelers from the ISWI family were reported to assemble and ensure proper nucleosome density and generate even spacing between nucleosomes in every eukaryote. Accordingly, *A. thaliana* ISWI proteins CHROMATIN-REMODELING 11 and 17 (CHR11 and CHR17) were reported to execute in the genome-wide distribution of nucleosomes [46]. ISWI proteins contain three domains, the HAND, SAINT, and SLIDE domains responsible for nucleosome binding. The SLIDE domain provides an interaction site to several DDT domain containing "accessory proteins" that convey specificity to the ATPase motor function of the ISWI remodeler [47]. ISWI complexes are comparatively smaller than other remodelers, consisting of approximately 2-4 subunits. Three types of ISWI remodeling complexes were identified by

immunoprecipitation coupled mass spectrometry (IP-MS), including CHR11/17-RLT1/2-ARID5 (CRA), CHR11/17-DDP1/2/3-MSI3 (CDM), and CHR11/17-DDR1/3/4/5/DDW1 (CDD) [48]. CRA complex was found to promote floral transition and stamen development [48,49], and DDP1 from the CDM complex is suggested to take part in retrograde signaling between the chloroplast and nucleus [50]. Information on the CDD type complexes was sparse, but recent findings suggest that DDR4/5 in combinations with CHR11 may promote the reactivation of silenced genes [51]. However, the role and biochemical mechanisms of these ISWI remodeler complexes and their uncharacterized accessory proteins require further studies.

1.2 Studying chromatin by In vitro chromatin assembly methods

Chromatin is a highly complex polymer made up of core canonical histones and histone variants, harboring histone modifications and non-histone chromatin binding proteins. While recent advancements in technology provide better insight into the workings and properties of in vivo chromatin. These techniques include chromatin immunoprecipitation coupled with sequencing (ChIP-Seq) that can identify the genomic DNA derived from the regions of chromatin enriched with specific histone modifications, histone variants, and other chromatin-associated proteins using specific antibodies. Moreover, nuclease coupled with sequence, such as DNase I sequence, can determine nucleosomal and non-nucleosomal DNA in the whole genome. Mass Spectrometry (MS) is another advanced approach, which is often used to identify post-translational modifications, histones, or chromatin-associated proteins [52,53]. Biochemical analysis of chromatin structure, dynamics and function is indispensable to dissect the individual function of canonical and variant histones, post-translational modifications, and chromatin-modifying enzymes. To define chromatin components for biochemical studies, in vitro reconstituted chromatin offers an excellent option.

In cells, *de novo* chromatin assembly is a stepwise process starting with the deposition of an H3-H4 dimer or tetramer. The H3-H4 tetramer, which wraps about 70 bp of DNA creates a stable intermediate product referred to as the tetrasome. Following the tetrasome formation, two H2A-H2B dimers are deposited to complete the nucleosomes. Histone deposition is mediated by histone chaperones, which may

be general or specific for the deposition of certain histone variants. Apart from nucleosome assembly, nucleosome disassembly can also be mediated by histone chaperones [54].

Histones are small basic proteins with a high abundance of lysine and arginine residues, that attract the negative sugar-phosphate backbone of the double-stranded DNA. If histone proteins and DNA are mixed in physiological ionic conditions, histone-DNA precipitates due to unspecific interactions [55]. The formation of properly folded nucleosomes requires the addition of histone chaperones and/or ATP-dependent chromatin remodeling factors, that shield histones, preventing unspecific interactions with the DNA. This method is rather laborious, and it requires many steps including purification and refolding of histones and chromatin assembly factors. Hence the most widespread *in vitro* nucleosome assembly method is the salt-dialysis method, which utilizes NaCl as a "histone chaperone" [56]. While this method yields biochemically pure nucleosomes in large quantities that may be well suited for structural studies, the assembly conditions are artificial. Refolded bacterially expressed histone octamers are mixed in a high salt buffer ~2M NaCl with a short DNA sequence selected for the optimal positioning of a nucleosome and dialyzed against a low salt buffer gradually or in a stepwise manner up to <150 mM NaCl [57]. The slow change in the ionic conditions allows nucleosome assembly in the structurally most favorable conformation, which results in the properly folded nucleosome. However, the conventional salt dialysis chromatin assembly method prevents the observation of natural phenomena occurring at the level of nucleosome assembly including screening for nucleosome assembly factors. Additionally, while short chromatin (like mono and di nucleosomes) could be a template for several applications, it cannot entirely represent the behavior of long nucleosome arrays found in the native chromatin in living cell nuclei.

2 CHAPTER 2: In vitro co-expression chromatin assembly and remodeling platform for plant histone variants

2.1 Abstract

Histone variants play a central role in shaping the chromatin landscape in plants, yet, how their distinct combinations affect nucleosome properties and dynamics is still largely elusive. To address this, a novel chromatin assembly platform for *Arabidopsis thaliana* was developed using wheat germ cell-free protein expression. Four canonical histones and five reported histone variants were used to assemble twelve *A. thaliana* nucleosome combinations. Seven combinations were successfully reconstituted and subjected to remodeling assay using a putative chromatin remodeling complex. Furthermore, two nucleosome combinations, which failed in the initial reconstitution reaction, were assembled by co-expressing a histone chaperone. Overall, the current study provides a novel method to elucidate the formation and function of a diverse range of nucleosomes in plants.

List of Abbreviations

CDD, CHR11-DDR4 complex

CHR11, CHROMATIN REMODELING11

DDR4, DDT domain-containing protein 4

MNase, Micrococcal Nuclease

NAP1;3, Nucleosome Assembly Protein 1;3

NCP, Nucleosome core particle

NRLs, Nucleosome repeat lengths

NSI, Nucleosome spacing index

2.2 Introduction

The eukaryotic genome is compacted and organized into chromatin; a long-range nucleoprotein complex made up of repeating units of nucleosomes. A nucleosome consists of a histone octamer, comprising two copies each of canonical histones H2A, H2B, H3, and H4, and approximately 147 bp DNA, which wraps around the octamer in ~1.7 turns [1]. In addition to a higher-order DNA packing, chromatin functions in various cellular processes, including transcriptional regulation, replication, DNA repair, and others, through epigenetic modifications of both DNA and histones and the incorporation of histone variants into nucleosomes [5,8,9]. The deposition of histone variants conveys unique properties to nucleosomes that affect chromatin stability [12], selectivity to epigenetic enzymes [13,14], and higher-order chromatin structure [15,16], which in turn, greatly diversifies the chromatin landscape.

In *Arabidopsis thaliana*, there are four types of histone H2As, including the canonical H2A, and three variants, H2A.X, H2A.W, and H2A.Z. Variants differ from canonical H2A by the length or unique motifs of their C-terminal tails, and conserved residues in their histone fold domains [22,24]. H2A.X is known to play a critical role in signaling DNA damage response through C-terminal phosphorylation [9,10,25]. H2A.Z is reported to be mainly associated with transcriptional regulation in plants [26,27] and other eukaryotes including humans [28] and yeast [29]. H2A.W is a plant-specific histone variant, found almost exclusively in heterochromatin [15] and acts in DNA damage response in these highly condensed regions [9]. *Arabidopsis* H2Bs are classified into three groups based on their amino acid sequences and expression patterns [20]. Class I H2Bs, wherein H2Bs with canonical histone-like features are categorized, mainly function in somatic tissues, and show peak expression levels in actively dividing cells [20,30]. Class II/III H2Bs are known to control chromatin structure in reproductive tissues [20,31]. However, our current understanding of the physiological functions of H2B variants in plants is still limited. Three main histone H3s are known in *A. thaliana*, including the canonical H3.1 and the variants H3.3 and CENH3, all of which are known to incorporate into different regions of the genome [32,33]. The functions of H3.3 and centromeric H3 are well-conserved across eukaryotes, where H3.3 has been linked to transcriptional activation [34], and the centromeric H3 is reported to aid in chromosome segregation through directing

kinetochore assembly [36,37]. In contrast, there is no histone H4 variant in *A. thaliana*. The histone variants used in this study and their reported characteristics are listed in Table 1.

In plants and other eukaryotes, histone variants may form over hundreds of different types of nucleosomes [8]. For instance, the unstable H2A.Z and H3.3 double variant-containing nucleosomes are specifically enriched at the promoter and enhancer regions, implying their role in promoting gene expression in humans [58]. Recent studies on *A. thaliana* revealed that H2A variants form homotypic nucleosomes, including two copies of the same variant types [12], while H3.1 and H3.3 seem to be present in both hetero- and homotypic nucleosomes in the genome [59]. Despite the accumulated knowledge, the physiological roles of such combinations have not been reported in plants, and it is not entirely clear which combinations of histone variants might even be capable of forming nucleosomes. Thus, how the above-mentioned histone variant-containing nucleosomes affect the function of remodeling or histone chaperone activity have not been assessed biochemically.

To this end, twenty-four *A. thaliana* nucleosome combinations from canonical and variant histones were reconstituted using the wheat germ cell-free co-expression chromatin assembly system described in recent studies [60–62]. Among the 24 tested combinations, seven types of nucleosomes were successfully reconstituted. Furthermore, these seven types of variant-containing nucleosomes were subjected to remodeling reactions using an ISWI-type remodeling complex, comprising CHR11 and DDR4 [48,63]. At last, two additional types of nucleosomes were successfully assembled by a co-expressing Nucleosome Assembly Protein 1;3 (NAP1;3), *in vitro*.

Table 2.1. Summary of histone variants in A. thaliana used in this study and reported functions.

Core histone	Variant type	Genes name	NCBI RefSeq	Characteristics	References
H2A	H2A	HTA10	NM_103984.4	S-phase linked expression, uniformly marks gene bodies excluded from heterochromatin	[15]
	H2A.X	HTA3	NM_104344.2	abundant in chromatin, involved in DNA repair in euchromatin	[9,15]
	H2A.W	HTA6	NM_125380.4	localizes in heterochromatin, enhances chromatin condensation through a higher propensity to promote fiber-to-fiber interactions, maintains transposon silencing	[9,15,64]
	H2A.Z	HTA9	NM_104152.4	enriched at the TSS of expressed genes, enriched in gene bodies of response genes	[15]
H2B	Class I	HTB9	NM_114467.4	mostly localize to gene bodies and mainly expressed in somatic tissues	[20]
	Class II	H2B7	NM_111782	exhibits cell or cell cycle specific expression patterns, expressed mostly in reproductive tissues	[20]
H3	H3.1	HTR2	NM_100790.3	S-phase linked expression, enriched in silent areas of the genome, and associated with heterochromatin marks	[32]
	H3.3	HTR5	NM_001342565.1	predominantly localized towards the 3' end of genes and is positively correlated with gene expression, excluded from heterochromatin, all genes code for identical proteins, suggested to have a role in post embryonic development in seed	[32,43]
	CENH3	HTR12	NM_001331269.1	marks centromeres, essential in kinetochore formation	[37,65]
H4	H4	HIS4	NM_128434.4	S-phase linked expression, uniformly marks gene bodies	

2.3 Materials and Methods

Gene amplification by PCR using cDNA library. All targets for histones (except H2B.7, which was received from Semmelweis University), remodeling factors, and histone chaperone were amplified from *A. thaliana* cDNA libraries using reverse and forward primers (Fasmac, Atsugi, Japan) with overhangs designed for ligation independent cloning, listed in Supplementary Table 1 [66]. PCR products were purified by PEG precipitation (26% ($w \cdot v^{-1}$) PEG 8000, 6.5 mM $MgCl_2$, 0.6 M sodium acetate, pH 7), followed by ethanol precipitation.

Ligation independent cloning. Target genes were cloned into pEU0-E01-LICNot vector - designed for ligation independent cloning [66]. Briefly, the pEU0-E01-LICNot vector was linearized by *Ssp*I restriction enzyme and purified by gel extraction (Kanto Chemicals, Tokyo, Japan). Overhangs were created on both PCR products and the vector by T4 polymerase (Toyobo, Osaka, Japan), in the presence of dCTP and dGTP. The vector and the inserts were incubated at room temperature for 20 minutes in a 1:3 to 1:5 molar ratio, then transformed into *Escherichia coli* JM109 (Takara, Shiga, Japan). The transformants were selected on LB-Ampicillin plates and correct insert sizes were verified by colony PCR using a plasmid-specific primer pair designed for colony PCR (Table 2.2). Positive transformants were cultivated in a liquid LB culture and plasmids were extracted by NucleoSpin Plasmid QuickPure™ (Marcherery Nagel, Düren, Nordrhein-Westfalen, Germany). Insert sequences were verified by DNA sequencing (Eurofins, Tokyo, Japan) using the primers utilized for colony PCR as well (Table 2.2).

Table 2.2. Primers designed for ligation independent cloning.

Primer name	Sequence
HTA10 forward	5'-**TACTTCCAATCCAATGCA**ATGGCGGGTCGTGGTAAAAC-3'
H2A10 reverse	5'-**TTATCCACTTCCAATG**TCAATCGTCTTCAGCAGATGGCT-3'
HTA3 forward	5'-**TACTTCCAATCCAATGCA**ATGAGTTCCGGCGCCGG-3'
HTA3 reverse	5'-**TTATCCACTTCCAATG**TTAAAACTCTTGAGAAGCAGATCCGATATCG-3'
HTA6 forward	5'-**TACTTCCAATCCAATGCA**ATGGAATCCACCGGAAAAGTG-3'
HTA6 reverse	5'-**TTATCCACTTCCAATG**TTAAGCTTTCTTTGGAGACTTGACTG-3'
HTA9 forward	5'-**TACTTCCAATCCAATGCA**ATGTCGGGGAAAGGTGCTA-3'
HTA9 reverse	5'-**TTATCCACTTCCAATG**CTATTCCTTGGCGGATTTGTTG-3'
HTB9 forward	5'-**TACTTCCAATCCAATGCA**ATGGCGCCGAGAGCAGAGAA-3'
HTB9 reverse	5'-**TTATCCACTTCCAATG**TCAAGAGCTTGTGAATTTGGTAACAGCC-3'
HTR2 forward	5'-**TACTTCCAATCCAATGCA**ATGGCTCGTACCAAGCAG-3'
HTR2 reverse	5'-**TTATCCACTTCCAATG**CTAAGCTCGTTCTCCTCTG-3'
HTR5 forward	5'-**TACTTCCAATCCAATGCA**ATGGCTCGTACTAAGCAAACAG-3'
HTR5 reverse	5'-**TTATCCACTTCCAATG**TTAAGCACGTTCTCCTCTGATC-3'
HTR12 forward	5'-**TACTTCCAATCCAATGCA**ATGGCGAGAACCAAGCATC-3'
HTR12 reverse	5'-**TTATCCACTTCCAATG**TCACCATGGTCTGCCTTTTC-3'
HIS4 forward	5'-**TACTTCCAATCCAATGCA**ATGTCAGGAAGAGGAAAAG-3'
HIS4 reverse	5'-**TTATCCACTTCCAATG**TCAACCACCAAATCCATATA-3'
CHR11 forward	5'-**TACTTCCAATCCAATGCA**ATGGCGAGAAATTCGAATTCC-3'
CHR11 reverse	5'-**TTATCCACTTCCAATG**TCATCTCATCGACAGGTGC-3'
DDR4 forward	5'-**TACTTCCAATCCAATGCA**ATGGGTTCCTCCTCCGA-3'
DDR4 reverse	5'-**TTATCCACTTCCAATG**TTATGAATTGTCTGTCTTATCATCA-3'
NAP1;3 forward	5'-**TACTTCCAATCCAATGCA**ATGAGCAACGATAAGGACAG-3'
NAP1;3 reverse	5'-**TTATCCACTTCCAATG**TTACTGTTGTTTGCATTCAGG-3'
Insert check forward	5'-CGATTTAGGTGACACTATAGAACTC-3'
Insert check reverse	5'-TATAGGAAGGCCGGATAAGACG-3'

***In vitro* transcription**. The mRNA transcription reaction was conducted, accordingly to the given protocol (CellFree Sciences, Yokohama, Japan). Briefly, 2 μg of plasmid construct was transcribed by SP6 polymerase in the presence of Ribonuclease inhibitor (Promega, Madison, WI) in a final volume of 20 μL reaction mixture for 4 hours at 37˚C. 0.5 μL of 12 $U \cdot \mu L^{-1}$ DNase I (Nippon Gene, Tokyo, Japan) was added and incubated for 30 minutes at 37°C, followed by acidic Phenol:Chloroform:Isoamyl alcohol (25:24:1, $v \cdot v^{-1}$) extraction and ethanol precipitation. The mRNA was resuspended in 10 μL nuclease-free water, and concentrations were measured by Denovix DS11 (Denovix, Wilmington, Delaware). The mRNAs coding all histones were mixed stoichiometrically for chromatin assembly reaction [61].

Cell-free expression of individual proteins. Wheat germ extract (WEPRO7240H, CellFree Sciences) was used for the translation reaction of all histones and chromatin factors accordingly to the given protocol. Briefly, 10 μL of ~1 $\mu g \cdot \mu L^{-1}$ mRNA transcript solution, 10 μL wheat germ extract, 0.8 μL 1 $mg \cdot mL^{-1}$ creatine kinase were mixed and carefully layered under 206 μL of 1xSUB-AMIX (CellFree Sciences) to create a bilayer in a sterile microplate well. The translation reaction was performed for 20 hours at 26°C.

Co-expression chromatin assembly. Chromatin assembly grade wheat germ extract (WEPRO7240Ch, CellFree Sciences) was used to co-express all core histone combinations using approximately 2 μg purified mRNA template for each histone as described in the previous study [60]. Briefly, purified mRNA mixture containing combinations of four canonical and/or variants of histone H2A, H2B, H3, and H4 was mixed with 5 μL wheat germ extract, 0.4 μL of 1 $mg \cdot mL^{-1}$ creatine kinase, 0.5 μL of 0.5 $\mu g \cdot \mu L^{-1}$ pBSK plasmid, 0.1 μL of 20 $U \cdot \mu L^{-1}$ Topoisomerase I (Takara, Shiga, Japan) and adjusted with nuclease-free water to 10.7 μL. The reaction mixture was carefully layered under 103 μL of 1xSUB-AMIX (CellFree Sciences) to create a bilayer in a sterile microplate well. The chromatin assembly reaction was performed for 4 hours at 26°C.

Supercoiling assay. 50 μL translation mixture was digested by 1 μL of 600 $U \cdot mL^{-1}$ Proteinase K (Wako, Osaka, Japan), and the plasmid DNA was purified by Phenol:Chloroform:Isoamyl Alcohol (25:24:1, $v \cdot v^{-1}$,

pH 8), followed by ethanol precipitation, and resolved in 6.5 µL HD buffer (25 mM HEPES, 1 mM DTT, pH 7.6) with the addition of a trace amount of Ribonuclease A (Macherey-Nagel GmbH & Co., Dueren, Germany). Samples were run on a 0.8% agarose gel in 0.5x TBE buffer and stained by ethidium bromide. The gel image was analyzed using the ImageLab Software (Version 6.1.0 build 7, Biorad, Hercules, California).

Nucleosome remodeling. The co-translational nucleosome assembly was conducted as described above, with the addition of ~6 µg CHR11 (RefSeq number: NM_111515.5) either with or without ~6 µg DDR4 (RefSeq number: NM_001332380.1) mRNAs. Due to the increase in the number of proteins that need to be co-expressed with the four histones, the assembly reaction was performed for 16 hours at 26°C. The translation mix was subjected to Micrococcal Nuclease assay.

Micrococcal Nuclease Assay (MNase assay). 100 µL translation mixture was supplemented with 2.5 mM $CaCl_2$ (final concentration) and digested by 0.1 $U \cdot \mu L^{-1}$ MNase (Takara, Shiga, Japan). at 37°C. 35 µL aliquots were taken at 1 and 3 minutes and the reactions were halted by adding 5mM EGTA. The DNA was purified as described above, and suspended in 4.5 µL HD buffer containing a trace amount of Ribonuclease A. The samples were analyzed on 2% agarose gel in 0.5x TBE buffer and visualized by SAFELOOK™ Red Nucleic Acid Stain (Wako, Osaka, Japan). The gel image was analyzed using the ImageLab Software (Biorad). The nucleosome spacing indexes were estimated accordingly to the previous study from the band intensity values of stained agarose gels [67].

NAP1;3-mediated nucleosome assembly. The co-translational nucleosome assembly was conducted as described above, with the addition of ~6 µg NAP1;3 mRNA (RefSeq number: NM_125077.4). The nucleosome assembly reaction was conducted for 4 hours at 26°C. 50 µL of the translation mixture was used for supercoiling and MNase assay as described above.

2.4 Results and discussion

2.4.1 mRNA transcription from plasmid constructs

Histone variants were amplified from *Arabidopsis thaliana* Col-0 Seedling cDNA library by PCR reaction with primers designed for ligation independent cloning. Amplified PCR products were cloned into pEU-derived vectors designed for ligation independent cloning and *in vitro* mRNA transcription. Ligation independent cloning method enables fast cloning without using site-specific restriction enzymes. Sequence verified *E. coli* transformants were cultivated in liquid culture and plasmid constructs harboring histones were extracted.

Histone mRNAs were transcribed from the plasmid templates by Sp6 polymerase. High-quality mRNA is a prerequisite for successful protein translation. Sp6 polymerase transcribes long mRNA templates that stabilize the transcripts even in the absence of a polyA tail. The Sp6 polymerase is removed easier at the replication origin on the pEU plasmid, creating periodic lanes on the agarose gels corresponding to one to four rounds of mRNA [68] (Appendix Fig. S.1).

2.4.2 Cell-free synthesis of canonical histones and variants

Each *A. thaliana* canonical histone, H2A, H2B, H3.1, and H4, and their variants, including three H2A variants, H2A.X, H2A.W, and H2A.Z, two H3 variants, H3.3 and CENH3 were cell-free synthesized from crude mRNA templates, and all proteins were determined at their expected molecular weights by SDS-PAGE (Fig. 2.1**a**). The different molecular weights among canonical and variant H2As are consistent with their protein sequences (Fig. 2.1**b**). Compared to the canonical H2B.9, the N-terminal unstructured region of the H2B.7 variant has a deletion of 21 amino acids. Between H3.1 and H3.3, there are only four amino acid alterations, two in the N-terminal non-structural region and two in the α2 helix (Fig. 2.1**b**). While CENH3 has a large insertion of ~38 amino acids in its N-terminal tail, compared to H3.1, which is suggested to direct CENH3 loading into the centromeres of meiotic chromosomes [37].

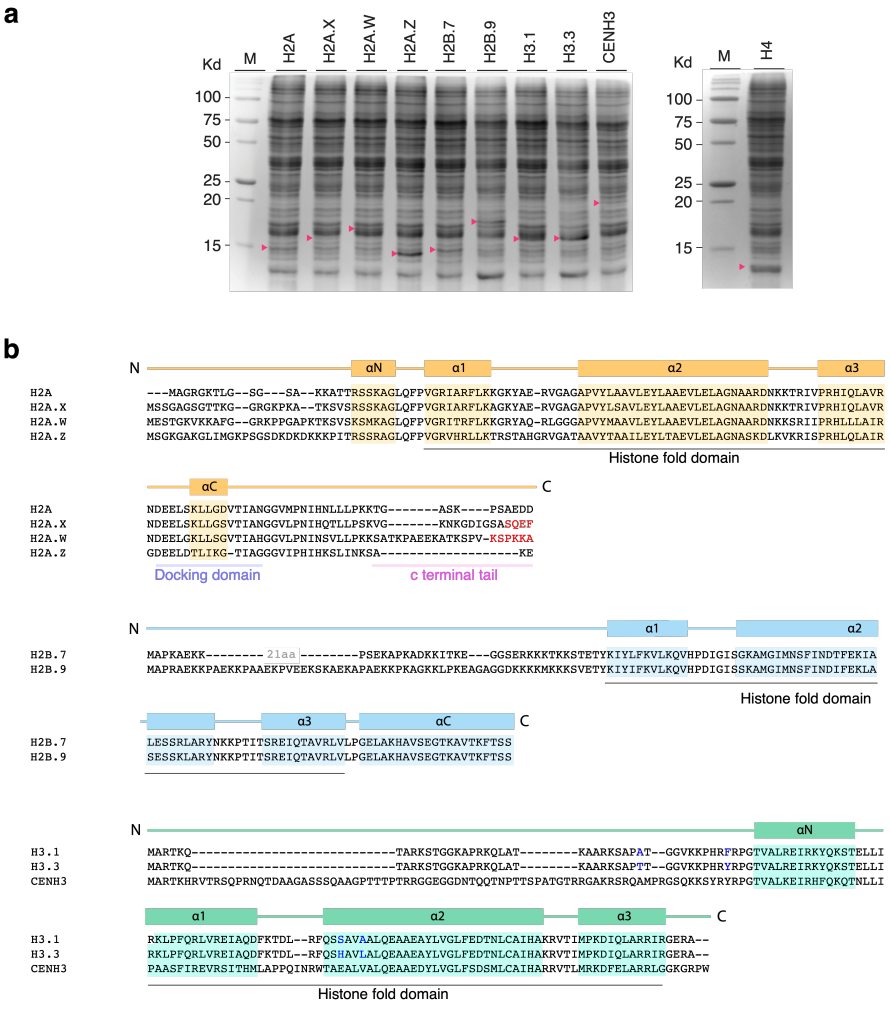

Figure 2.1. Canonical and variant histones synthesized by wheat germ cell-free synthesis.

(a) A. thaliana canonical histones, H2A, H2B.9, H3.1, and H4, and histone variants, H2A.X, H2A.W, H2A.Z, H2B.7, H3.3, and CENH3 were cell-free synthesized and analyzed on an 18% SDS-PAGE. The red triangles show the histone proteins. (b) Multiple sequence alignments of canonical histone H2A, H2B.9 and H3.1 with their variants used in this study are shown. The structural features of histone variants are provided (HistoneDB 2.0 database). Alpha helixes of the aligned histones are highlighted in orange, blue and green, respectively. The characteristic C-terminal tail motifs of H2A.X and H2A.W variants are indicated with red-colored fonts, and the characteristic substitutions between H3.1 and H3.3 are indicated with blue-colored fonts.

2.4.3 Reconstitution of histone variant-containing chromatin

To investigate if the wheat germ cell-free nucleosome assembly platform is suitable for the assembly of A. thaliana chromatin with histone variants, each of the twenty-four combinations of canonical histones and their respective variants (Table 2.1) was co-expressed in the presence of a relaxed pBSK plasmid DNA [60–62]. Previously, a slight endogenous chromatin assembly activity was reported in the wheat germ extract during the reconstitution of *Drosophila* chromatin [62]. As both wheat and *Arabidopsis* are plants, it was assumed that *Arabidopsis* chromatin could be efficiently reconstituted in the wheat germ-based co-expression chromatin assembly system.

For the chromatin assembly reaction, the *in vitro* transcribed histone mRNA templates were further treated with DNaseI enzyme, to get rid of the plasmid templates carrying histone genes in the transcription mixture, which would interfere with the downstream assays. DNaseI treated mRNA was extracted and purified by acidic phenol-chloroform extraction and ethanol precipitation. Histone mRNAs from each histone H2A, H2B, H3 and H4 families were mixed in equimolar ratios and co-expressed in the same translation reaction in the presence of pBSK plasmid (Fig 2.2). The supercoiling assay was used for the evaluation of the assembly of 24 chromatin combinations containing histone variants (Fig 2.3a). The supercoiling assay measures the linking number changes on a circular closed plasmid DNA by the electrophoretic migration speed. Relaxed open circular DNA migrates the slowest, while each twist on the plasmid, caused by torsional forces, will proportionally increase the electrophoretic migration speed of the plasmid. A single nucleosome core particle causes a linking number change of -1 on a circular plasmid DNA creating a distinct band on agarose gel; therefore, chromatin assembly can be monitored by the creation of supercoils in the presence of a plasmid relaxed by topoisomerase I [69]. Subnucleosomal species such as the H3-H4 tetrasome, an intermediate structure occurring during nucleosome assembly, can also cause changes in the linking number of DNA ($\Delta Lk = -0.73$) [70].

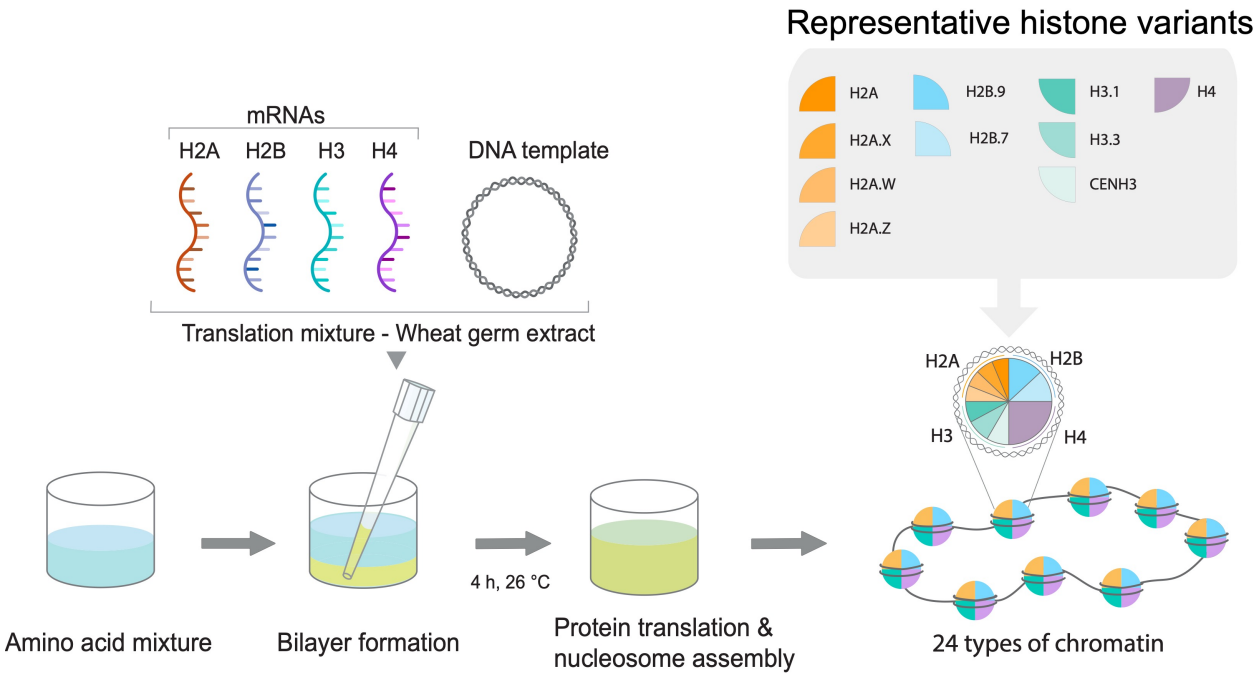

Figure 2.2. Schematic representation of the Wheat germ extract-based co-expression chromatin assembly platform for histone variant containing chromatin.

The wheat germ extract-based co-expression chromatin assembly platform is used for the simultaneous expression of the four core histone proteins from mRNA templates and chromatin reconstitution. Twenty-four combinations of histone variant coding mRNAs were mixed in the presence of a relaxed plasmid DNA and co-expressed in a 4 hour reaction.

To avoid false positive results caused by subnucleosomal species, the amounts of mRNAs encoding H3 and H4 were empirically set to a concentration that does not result in complete supercoiling by the formation of H3-H4 tetrasomes on the plasmid DNA in a 4-hour-long reaction (Fig. 2.3) [61]. The same amounts of H3-H4 mRNAs were used in the octameric nucleosome assembly reaction, with the addition of H2A and H2B mRNA. When compared to the H3-H4 control lane, the observed formation of supercoils in the presence of four types of H2As (H2A, H2A.X, H2A.W, and H2A.Z) and H2B should represent the efficiency of complete nucleosome formation on the circular DNA.

Out of the twenty-four nucleosome core particle (NCP) combinations, seven NCPs showed apparent supercoiling formation, suggesting that this method suites to reconstitute *A. thaliana* chromatin with canonical histones and several histone variants (Fig. 2.3b). H2B.7 did not show improvement in supercoiling compared to the control H3-H4 lane in any combinations, therefore the 12 combinations containing this variant were omitted in the following experiments.

Regarding H2A and H2A.X, which showed supercoiling regardless of the H3/H4 combinations, including H3.1/H4, H3.3/H4 and CENH3/H4, our observation is consistent with a previous *in vivo* genome-wide profiling study in *A. thaliana,* where H2A.X was shown to be ubiquitously found throughout the genome with different types of histone H3s [15]. H2A.W showed substantial supercoiling in the presence of H3.1 but not with H3.3. Previous findings showed that H2A.W is strictly deposited in the heterochromatin regions and the pericentromeric regions with H3.1 [15], whereas H3.3 is reported to be depleted in the heterochromatin regions in *A. thaliana* [32,34]. The described two amino acid difference in the α2 helix of H3.3 compared to H3.1 (Fig. 2.1**b**) might alleviate nucleosome formation with H2A.W, which needs further confirmation. H2B.7, a class II type H2B variant, did not form nucleosomes in the four-hour reaction. This variant is barely detectable in somatic tissues but shows specific expression in the sperm and the beginning stages of embryo development [20]. Due to these specific expression patterns, the deposition of this variant into nucleosomes may require different chromatin formation factors that the wheat-germ extract lacks. Exploring the role of deposition of H2B variants in class II can be an interesting objective of future studies. Additionally, the combination of CENH3 and H2A.W, H2A.W/CENH3 NCP,

were not supercoiled. Incompatibility between these variants may arise from the extended C-terminal tail of H2A.W that may be structurally unfavorable for CENH3 (Fig. 2.1**b**). None of the combinations containing H2A.Z were assembled in our system, suggesting that H2A.Z might require specific factors for its deposition into chromatin, such as a histone chaperone [63,71]. The absence of H2A.Z deposition in our current protocol enables us to screen for factors that enhance H2A.Z incorporation through a co-expression manner, which will be addressed later.

Supercoiling assay results were further supported by a partial Micrococcal Nuclease digestion (MNase) assay (Fig 2.4b). MNase is an endonuclease that cleaves free linker DNA between nucleosomes, but its activity is greatly hindered in DNA regions associated with the core histone octamer (Fig2.4a). Thus, the chromatin subjected to a limiting MNase digestion generates chromatin fragments of varying sizes from mono-, di-, tri to oligo nucleosomes. Deproteinized DNA fragments can be analyzed by gel electrophoresis revealing an "MNase ladder" corresponding to the aforementioned nucleosomal fragments. Twelve combinations, from here on H2A/H2B.9/H3.1/H4 (NCP), H2A.X/H2B.9/H3.1/H4 (H2A.X NCP), H2A.W/H2B.9/H3.1/H4 (H2A.W NCP), H2A/H2B.9/H3.3/H4 (H3.3 NCP), H2A.X/H2B.9/H3.3/H4 (H2A.X/H3.3 NCP), H2A/H2B.9/CENH3/H4 (CENH3 NCP), and H2A.X/H2B.9/CENH3/H4 (H2A.X/CENH3 NCP), were digested by MNase in a time course of 0, 1, 3 and 6 minutes and analyzed on agarose gel (Fig 2.4b). Longer MNase digestion time reveals an approximately ~150 bp DNA fragment inn all samples, which indicates the presence of properly formed nucleosomes. The seven combinations that showed positive results by supercoiling assay in Figure 2.3, including NCP, H2A.X NCP, H2A.W NCP, H3.3 NCP, H2A.X/H3.3 NCP, CENH3 NCP and H2A.X/CENH3 NCP, displayed extensive DNA ladders. Among these combinations, CENH3-containing combinations displayed distinct ladders compared with other variants, while H3.3-containing variants displayed less prominent MNase ladders with a smear in the background. These differences may be attributed to the specific features conveyed by histone H3 variants in the chromatin contexts. Nucleosome-sized bands up to dinucleosomes were observed among the five combinations that did not exhibit supercoiling (H2A.Z NCP, H2A.W/H3.3 NCP, H2A.Z/H3.3 NCP, H2A.W/CENH3 NCP and H2A.Z/CENH3 NCP). However, the MNase sensitivity of these samples was

high, indicating a low assembly quality with long stretches of nucleosome-free naked DNA. These results are in agreement with the results of the Supercoiling assay.

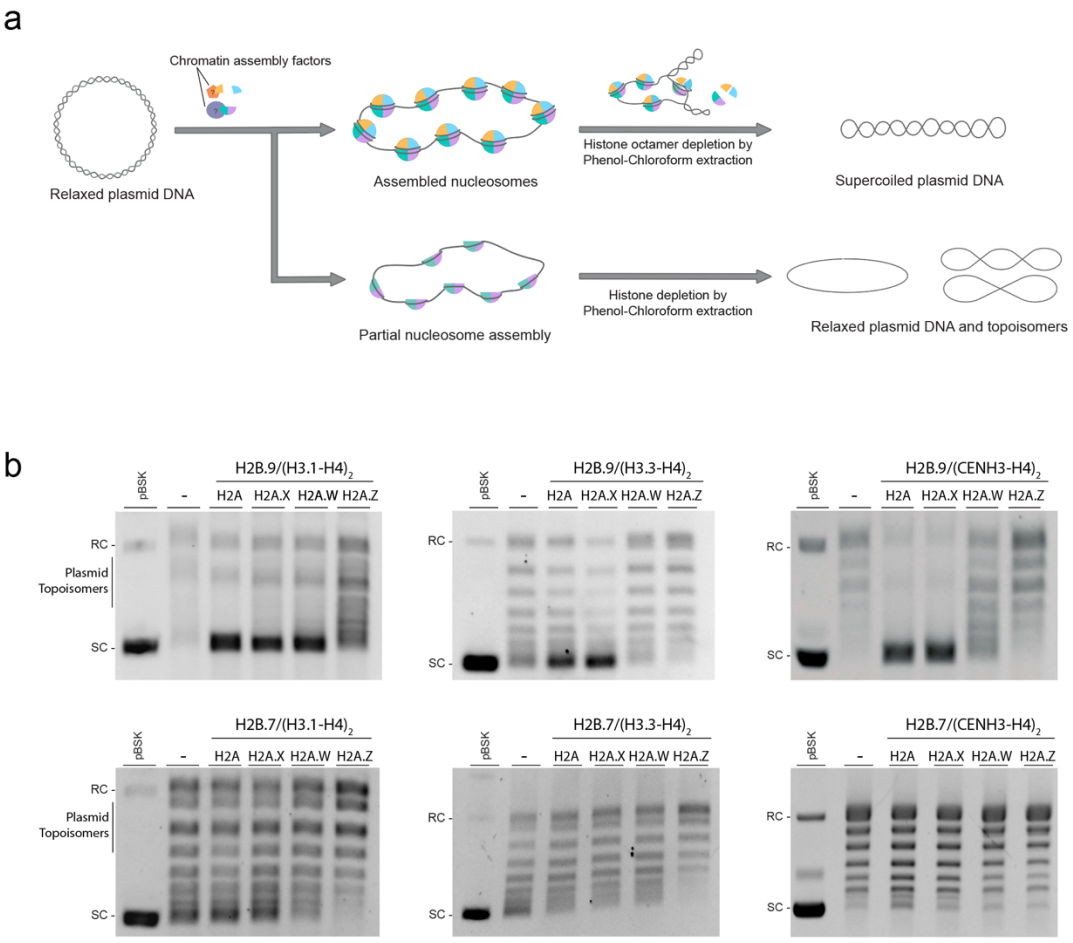

Figure 2.3. DNA supercoiling assay of *Arabidopsis* chromatin combinations.

(a) Schematic representation of the principles of supercoiling assay. **(b)** Supercoiling assay results of twelve nucleosome combinations are shown. Deprotonated plasmid samples were separated on a 0.8% agarose gel. The first lane contains untreated, supercoiled pBSK plasmid as a DNA topology marker. The second lane only contains the respective $(H3.1\text{-}H4)_2$, $(H3.1\text{-}H4)_2$, or $(CENH3\text{-}H4)_2$ tetrasomes in the presence of relaxed pBSK. The rest of the lanes contain the indicated nucleosome samples. The SC and RC indicate the formation of supercoiling and relaxed circular plasmid, respectively.

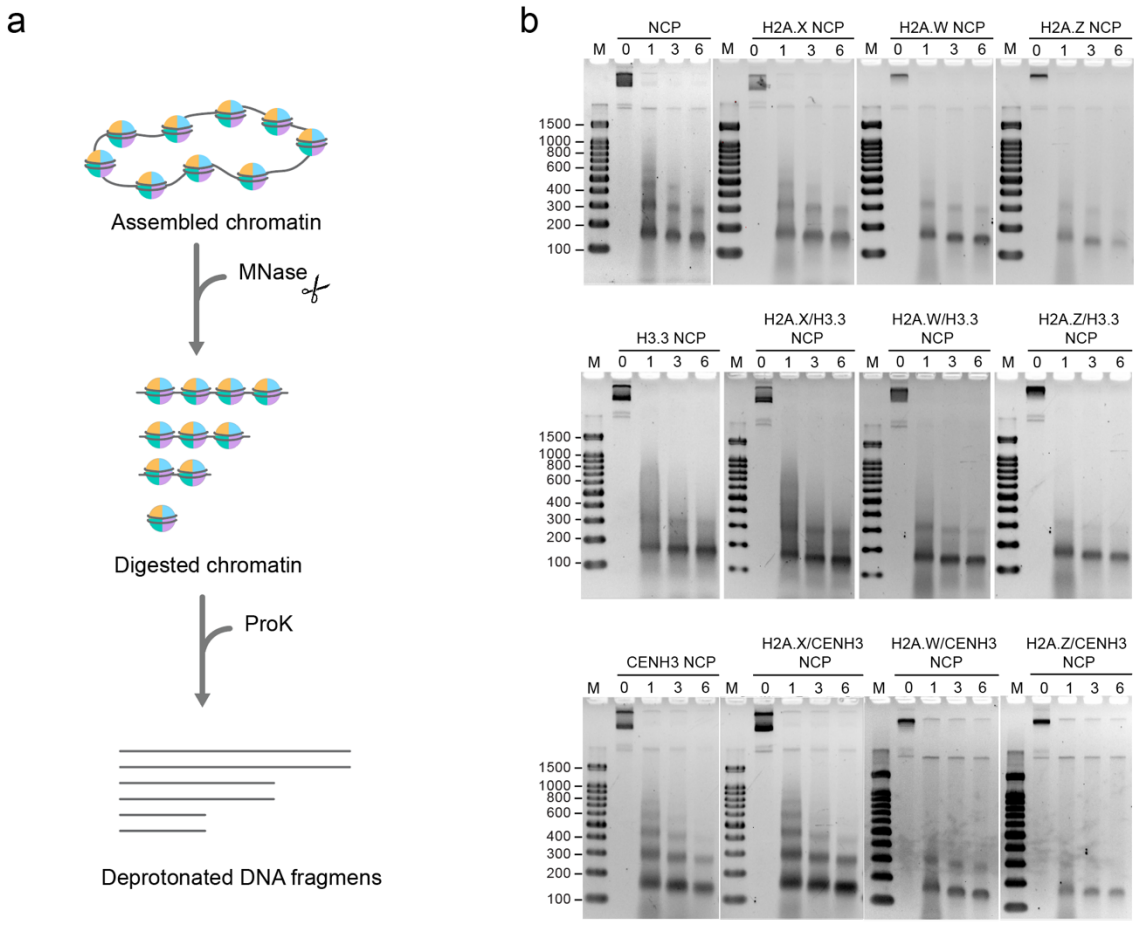

Figure 2.4. MNase assay results of *Arabidopsis* chromatin combinations.

(a) Schematic representation of the principles of MNase assay. (b) Gel electrophoresis results of the MNase assays of the twelve chromatin combinations. Each combination was digested by the same concentration of MNase enzyme for increasing time increments of 0, 1, 3 and 6 minutes. DNA fragments were resolved on a 2% agarose gel and stained by GelRed.

2.4.4 Remodeling of histone variant-containing chromatin with a plant ISWI complex

The seven successfully reconstituted chromatin combinations were further tested as substrates for chromatin remodeling activity. Two imitation switch (ISWI) ATPase chromatin remodelers, CHR11 and CHR17, are known to facilitate nucleosome sliding and act redundantly to each other in *A. thaliana* [46,72]. In recent studies, these ISWI-type chromatin remodelers were reported to form complexes with single or multiple accessory proteins from the DDT-domain protein family [47,48]. Accessory proteins in the DDT-related (DDR) subfamilies, including DDR1, DDR3, DDR4, and DDR5, were reported to form a heterodimer with either CHR11 or CHR17 [48]. Among DDRs, DDR4 was highly enriched when CHR11 or CHR17 was used as bait for immunoprecipitation followed by mass spectrometry in the *A. thaliana* cell lysate [63]. Hence, CHR11 and DDR4 were chosen for the remodeling assay for the seven successfully reconstituted chromatins. The CHR11 and DDR4 proteins were confirmed to be synthesized by the wheat germ cell-free reaction with their expected molecular weights (Fig. 2.5**a**). Either CHR11 or CHR11/DDR4 (CDD complex) was co-expressed with seven types of variant-containing nucleosomes followed by partial Micrococcal Nuclease assay (Fig. 2.5**b**). The nucleosome repeat lengths (NRLs) were estimated for the reconstituted chromatins in the absence or presence of remodeling activity from the MNase ladder corresponding to di-nucleosomes (n=3). In the absence of CHR11 and DDR4 mRNAs, the average NRLs ranged from ~145 bp to 160 bp among canonical and six variant histone containing-chromatins (Table 2.3). The canonical (NCP) and H2A.X-containing (H2A.X NCP) chromatins showed NRLs of ~147 bp, while the H2A.W NCP showed longer NRLs of ~157 bp, consistent with the previous report that H2A.W protects a longer stretch of DNA through its C-terminal KPSKK motif [12]. In H3.3-containing two chromatin combinations (H3.3 NCP and H2A.X/H3.3 NCP), apparent NRLs were ~150 bp. In contrast, the estimated NRLs of CENH3 NCP and H2A.X/CENH3 NCP of ~145 bp were slightly shorter than other variants. In humans, CENP-A nucleosomes were found to have looser contact with the DNA at the entry-exit site [36], resulting in high MNase sensitivity [73], which could explain the observed shorter NRLs in two CENH3-containing chromatins.

The NRLs of all nucleosome combinations appear to be longer when CHR11 or both CHR11 and DDR4 proteins (CDD complex) were co-expressed in the chromatin assembly reaction, indicating the remodeling function of CDD complex (Fig. 2.5**b** and Table 2.3). Additionally, compared to the control reaction, the CENH3-containing two nucleosomes, CENH3 NCP and H2A.X/CENH3 NCP, showed apparently longer NRLs of ~7 bp in the presence of the CDD complex. The longer NRLs found in the remodeled chromatins are consistent with the reported "ruler" function of the ISWI remodeling complex, which defines equal linker distances between neighboring nucleosomes in the chromatin context [74,75]. Our results further suggest that the length of the "ruler" might differ depending on the existing histone variants in chromatins.

To gain further insight into the remodeling function that would provide regular linker distances among the nucleosomes in the remodeled chromatins, the nucleosome spacing index (NSI) was estimated from three independent MNase agarose gel results [67] (Fig. 2.5b). The three MNase gel replicas and their calculated NSI values are available in Appendix Fig S.4-6. Irregularly spaced nucleosomes will generate a pool of DNA fragments with random lengths, which appears as a background behind the MNase ladder or a smear. The NSI evaluates the periodicity of nucleosomes, by the intensity of the nucleosome bands and the background signal (Fig. 2.5c). The estimated NSI of each combination was used to calculate the fold change in NSI in the presence of either CHR11 or the CDD complex relative to the control, in which no remodeling factors were added (Fig. S.3). In the canonical NCP, with the co-expression of both CHR11 and DDR4 proteins, the NSI values resulted in a more than two-fold increase compared to the absence of remodeling complex. In comparison, only a slight increase was seen for the H2A.X NCP and H2A.W NCP. Interestingly, the fold change of NSIs in H3.3 NCP and H2A.X/H3.3 NCP in the presence of remodeling activity was more than 2.5-fold greater than the control. These results suggest that the CDD remodeling complex indeed defines the regular spacing in the remodeled chromatin combinations. In contrast, the effect of CDD complex on NSIs was negligible in the CENH3-containing combinations, CENH3 NCP and H2A.X/CENH3 NCP. The little to no change in the NSIs in both CENH3-containing chromatins is likely attributed to the well-defined MNase ladders, even in the absence of the remodeling activity. To note, the

remodeling activity observed when only CHR11 was co-expressed in our system was considerably lower compared to the values of estimated NRLs and NSIs in the presence of DDR4, especially for H3.3 containing chromatins. Thus, CHR11 needs an accessory protein to efficiently remodel chromatins (Fig. 2.5). Furthermore, these results suggest that the remodeling complex consisting of CHR11 and DDR4 provide different NRLs and regular spacings depending on the types of chromatins containing different histone variants. Our findings support the notion that ISWI-type remodelers can act preferentially toward variant-containing nucleosomes [76].

To note, a recent paper published on DDR4 function provided *in vivo* evidence of the remodeling activity of the CHR11-DDR4 complex and proposed its role in the reactivation of silenced heterochromatic regions by reducing nucleosome occupancy [51]. While the biggest increase in NSI was observed in the H3.3 containing combinations that generally occupy euchromatic regions, variant combinations associated with heterochromatin were targeted by the CDD complex as well. What mechanisms direct DDR4 to heterochromatic chromatin regions should be addressed in future studies.

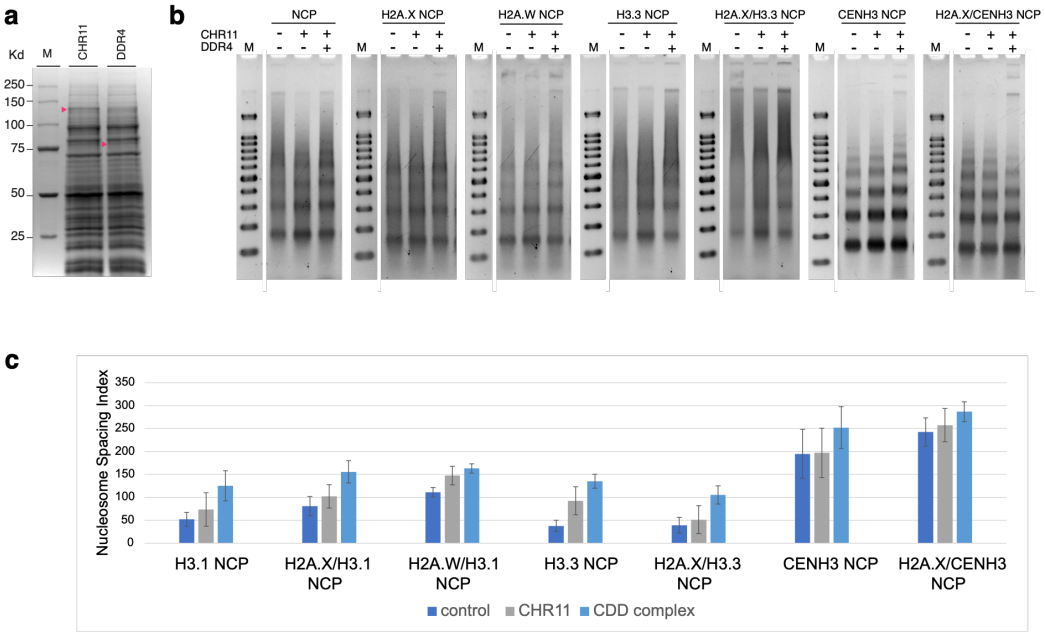

Figure 2.5. The effect of ISWI remodeling complex on seven reconstituted chromatin combinations. (a) The cell-free synthesized CHR11 and DDR4 proteins are shown. (**b**) The mRNAs (CHR11 individually or along with DDR4) were co-expressed during the chromatin assembly reaction along with the control reaction without CHR11 and DDR4 mRNAs, followed by partial MNase digestion. (**c**) Nucleosome spacing indexes (NSIs) were calculated from the densitometry images of three MNase technical replicates. The error bars indicate the standard deviation between the three technical replicas. Uncropped agarose gel images are provided in Appendix Fig. S4-6.

Table 2.3. Effects of the CDD remodeling complex on the nucleosome repeat lengths of seven nucleosome types.

Nucleosome combinations	No remodeling activity (bp) [*1]	Presence of CDD complex (bp) [*1]
NCP	146±3.7	151±3.6
H2A.X NCP	148±3.2	152±2.2
H2A.W NCP	157±3.8	161±4.3
H3.3 NCP	149±7.8	153±3.9
H2A.X/H3.3 NCP	147±6.9	152±3.9
CENH3 NCP	146±2.1	154±0.7
H2A.X/CENH3 NCP	144±1.3	150±4

[*1] Average and standard deviation was estimated from 3 experimental replicas.

2.4.5 NAP1 mediated chromatin assembly of chromatin with histone variants

Out of twelve combinations of chromatins, five were not reconstituted (Fig. 2.4b). It was considered that these combinations can be used to test *A. thaliana* histone chaperone activity. One of the well-studied histone chaperones is *Drosophila* NAP1, and its function during chromatin assembly has been described [77]. In *A. thaliana*, there are four NAP1 homologs (AtNAP1;1–4) and two NAP1-Related Proteins (AtNRP1 and AtNRP2). Among these, NAP1;3 was shown to be the major isoform in *A. thaliana* [78]. Thus, we decided to examine whether NAP1;3 helps to assemble those five types of nucleosomes by co-expressing NAP1;3 in the chromatin assembly reaction.

As shown in Fig. 2.6**a**, NAP1;3 protein was synthesized with the expected molecular weight by the wheat germ cell-free system. NAP1;3 mRNA was co-expressed in the chromatin assembly reaction, alongside histone mRNAs. In the presence of NAP1;3, the supercoiling formation of two NCPs, H2A.Z NCP and H2A.Z/H3.3 NCP, were facilitated (Fig. 2.6**b**). The MNase assay results of these two combinations show more extensive MNase ladders on the agarose gel in the presence of NAP1:3 (Fig. 2.6**c)**. Higher molecular weight and periodic DNA fragments indicate higher protection against nuclease digestion and the presence of periodic nucleosome arrays, further supporting the improved supercoiling assay results in the presence of NAP1;3.

In contrast, the effect of NAP1;3 was limited for the other three combinations, H2A.W/H3.3 NCP, H2A.W/CENH3 NCP and H2A.Z/CENH3 NCP (Appendix Fig. S.2). These combinations may require additional factors, currently unknown, or these combinations of nucleosomes might not exist *in vivo*. From these results, it was concluded that the above-mentioned deficiency in H2A.Z/H2B deposition in the wheat germ cell-free reaction is aided by co-expressing NAP1;3 in the H3.1 and H3.3 nucleosomes. Consistently, NAP1 family chaperones have been shown to interact with several types of histone variants in an unselective manner. *In vivo* studies demonstrated that yeast NAP1 binds not only the canonical H2A/H2B but also H2A.Z/H2B dimers [79,80]. Additionally, the human homolog, hNAP1L, was co-purified with histone variants from cell extracts [81], indicating that NAP1 family chaperones play a part in histone variant trafficking and deposition. Thus, the determined *A. thaliana* histone chaperone activity for H2A.Z-

containing chromatins in this study suggests that the NAP1;3 facilitates H2A.Z/H2B deposition in plants, consistent with the reported NAP1 functions in other organisms.

Figure 2.6. NAP1;3-mediated nucleosome assembly of two H2A.Z containing nucleosomes, H2A.Z NCP and H2A.Z/H3.3 NCP.

(a) NAP1;3 was synthesized by the cell-free system, indicated by the red triangle on the SDS-PAGE gel. (b) Schematic representation of the experimental setup and supercoiling assay results of H2A.Z NCP and H2A.Z/H3.3 NCP assembled in the absence or presence of histone chaperone NAP1;3. (c) MNase assay results of H2A.Z NCP and H2A.Z/H3.3 NCP, assembled in the absence or presence of histone chaperone NAP1;3.

2.5 General discussion

Histone variants are key nucleoproteins in regulating chromatin dynamics and function and are indispensable for maintaining plant physiology, yet the current knowledge is still limited. To overcome the limitations of *in vivo* studies, a novel platform suitable to study plant histone variants and their interaction with several plant chromatin factors was developed and optimized.

In the present study, we investigated the chromatin assembly properties of the wheat germ-based chromatin assembly platform for assembling various histone variant-containing nucleosome combinations from *A. thaliana*. We tested the assembly of canonical histones, H2A, H2B, H3.1, and H4, and histone variants, including three H2A variants, one H2B variant, and two H3 variants, resulting in twenty-four combinations of nucleosomes. Seven combinations, including the canonical NCP, were successfully reconstituted in a co-expression manner using the wheat germ cell-free chromatin reconstitution system.

Assembled combinations were subjected to chromatin remodeling reaction by co-expressing components of an ISWI family complex (CHR11 and DDR4) in the same system. We could observe the effect of remodeling activity on different combinations of histone variant containing chromatins, which provided an interesting mechanistic insight into the relationship between histone variants and remodelers.

Furthermore, co-expressing a reported histone chaperone, NAP1;3, with two H2A.Z-containing chromatins, that were not assembled initially, we showed that NAP1;3 successfully facilitates the formation of chromatin. Studying the selective nature of histone chaperones toward certain combinations of histone variants could reveal currently undescribed chromatin regulatory pathways *in vivo.*

The majority of the studies on plant chromatin focus on improving our understanding of fundamental biological processes, but some researchers also see potential in emerging epigenetic-based strategies for crop breeding [82,83]. It has been widely acknowledged that epigenetic information can pass on heritable phenotypic traits to the next generations. Epigenetic marks may also hold as potential biomarkers for predicting the presence of a desired trait or predicting crop survival and performance within a plant population [84].

While the current method can offer a simple and direct approach to studying chromatin function *in vitro*, the limitations should be taken into consideration. Despite the wheat-germ extract-based protein expression system produces the highest yield among eukaryotic cell-free protein expression platforms [85], the amount of chromatin that can be produced by this system is considerably lower than other chromatin assembly approaches that utilize bacterially expressed histone proteins. Therefore, downstream applications that require a lot of proteins, such as structural studies, may be expensive.

In summary, the current method is a useful tool to assemble nucleosomes with various histone variants and to functionally determine the remodeling activities and the histone chaperone activities related to chromatin structure and physiological functions in plants.

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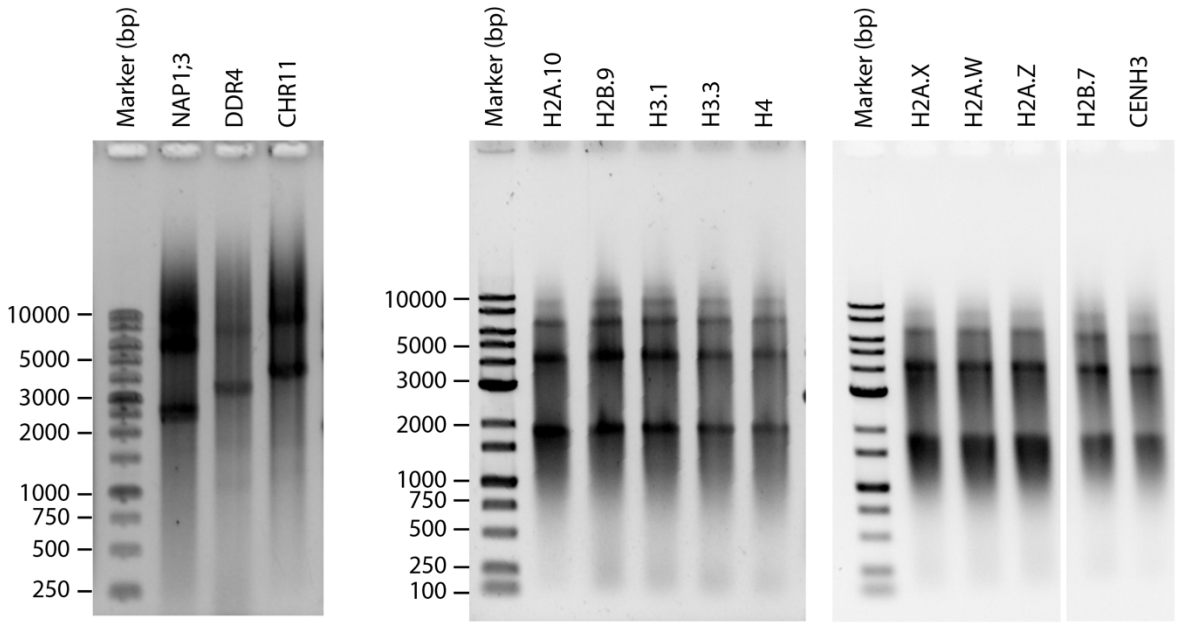
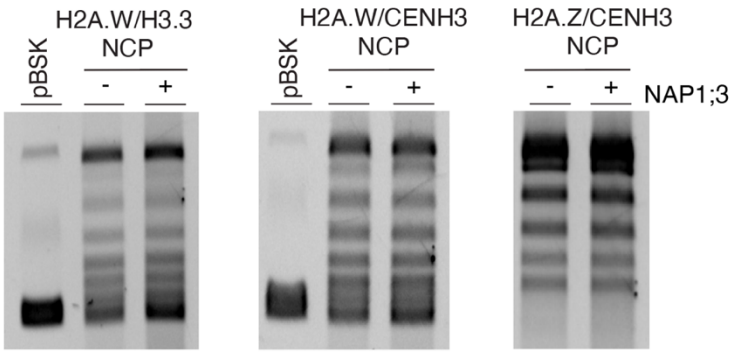
2.7 Appendix

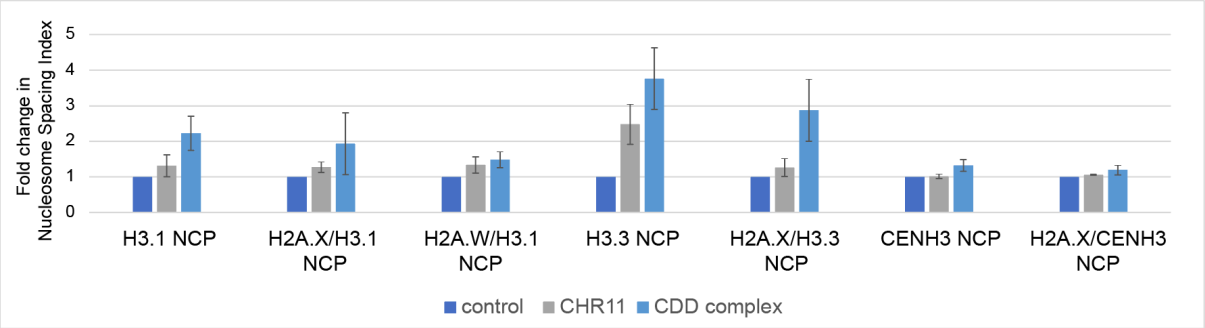


Appendix Fig. S.1. Electrophoresis of mRNA templates.

In vitro transcribed mRNAs of histones and remodeling factors were run on an agarose gel to confirm the integrity and quality of the transcripts. The three to four characteristic bands indicate high-quality mRNA templates.

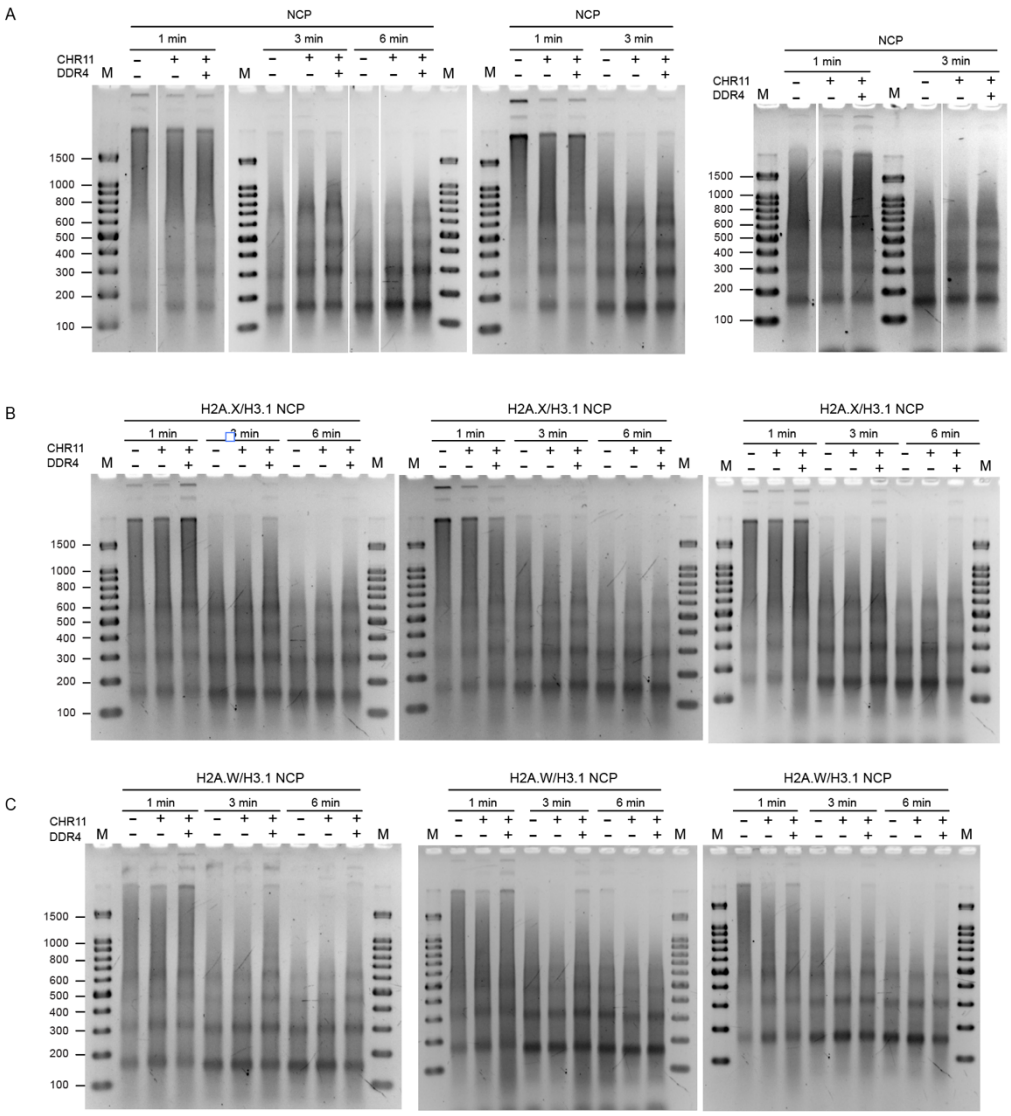


Appendix Fig. S.2. NAP1;3-mediated nucleosome assembly of three nucleosome combinations. Supercoiling assay results of NAP1;3-mediated chromatin assembly of unsuccessfully assembled variants. H2A.W/H3.3, H2A.W/CENH3, and H2A.Z/CENH3 NCPs were assembled in the presence of the NAP1;3 mRNA. The assembly of the three combinations was not facilitated by the addition of NAP1;3.

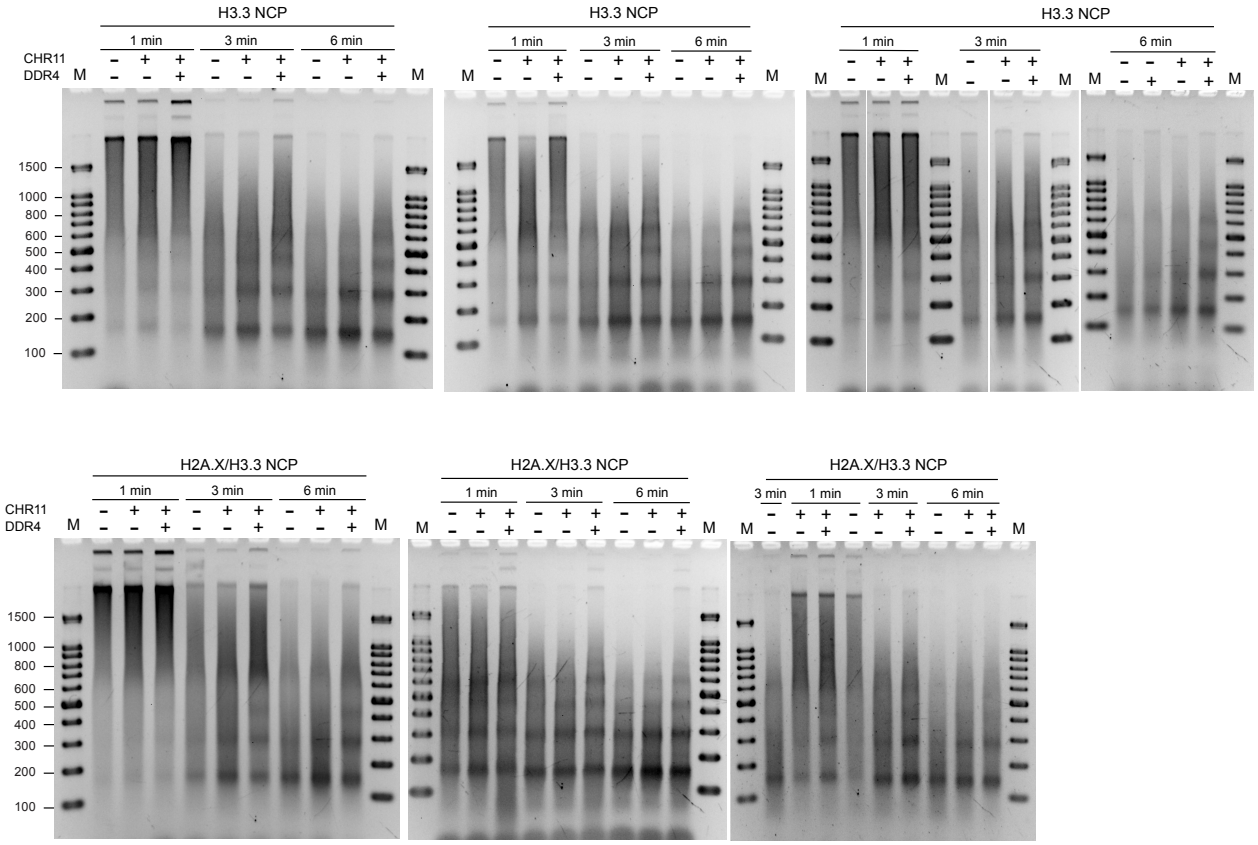


Appendix Fig. S.3. NAP1;3-mediated nucleosome assembly of three nucleosome combinations.

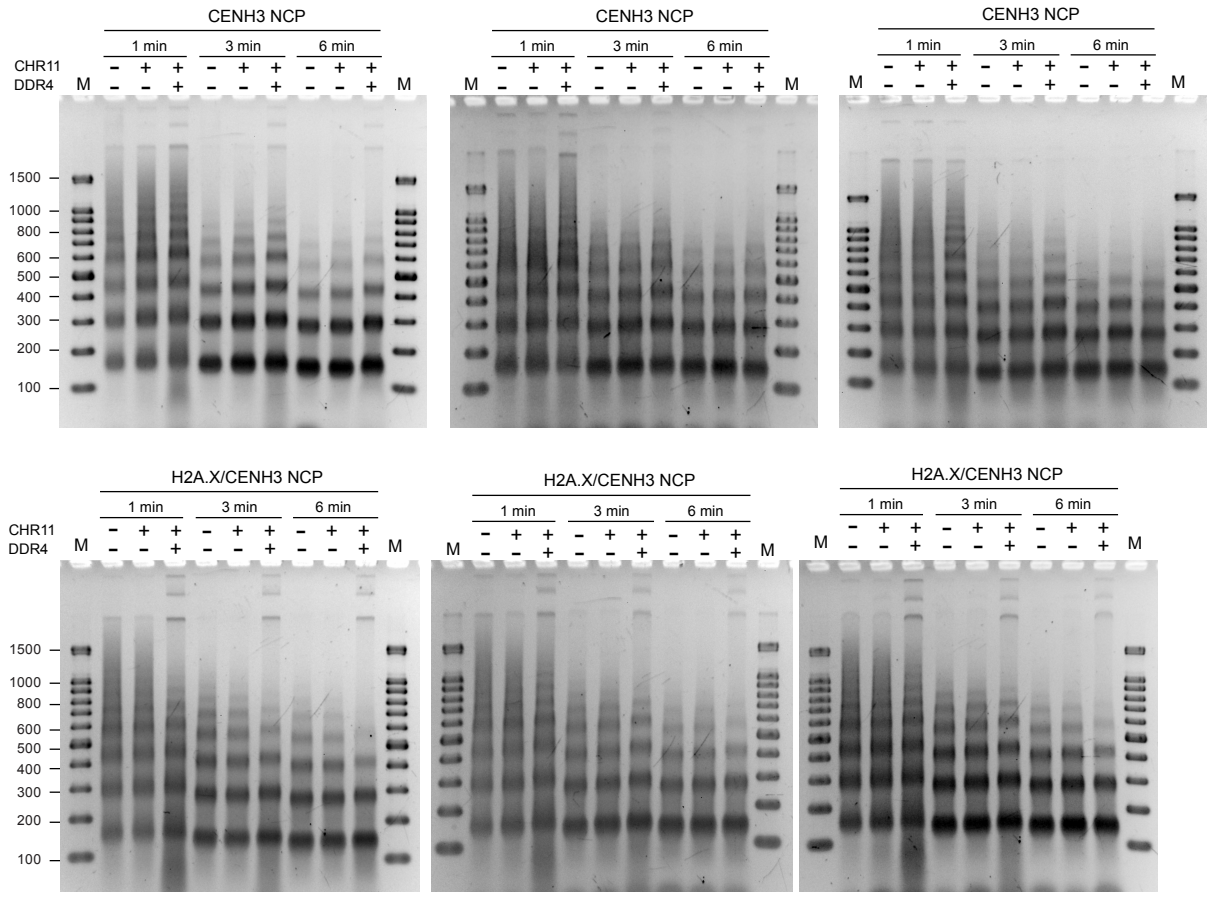
Using the calculated nucleosome spacing indexes, the fold change in NSIs were calculated from the presence of CHR11 or CDD complex relative to the control in each canonical and variants nucleosomes.



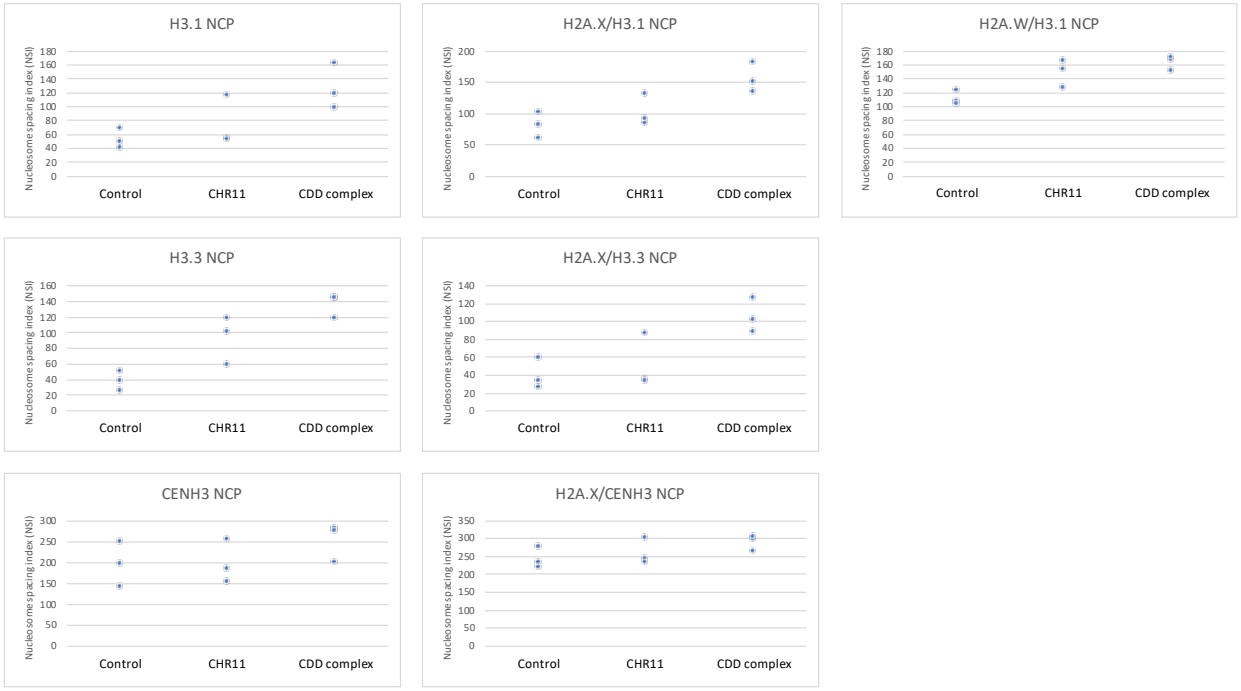
Appendix Fig. S.4. Uncropped MNase assay results of all three technical replicas of NCP, H2A.X NCP and H2A.W NCP templated chromatin remodeling experiments.



Appendix Fig. S.5. Uncropped MNase assay results of all three technical replicas of H3.3 NCP and H2A.X/H3.3 NCP templated chromatin remodeling experiments.



Appendix Fig. S.6. Uncropped MNase assay results of all three technical replicas of CENH3 NCP and H2A.X/CENH3 NCP templated chromatin remodeling experiments.



Appendix Fig. S.7 Scatter plots of the calculated NSIs of the seven tested NCP combinations.