Histone H3 and its Centromeric Variant Cse4 Co-Occupy the Centromeric DNA in *Saccharomyces cerevisiae*

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ABBREVIATIONS

A, Ala	alanine
A/T	adenine / threonine
aa	amino acid
Ade	adenine
AFM	atomic force microscopy
Amp	ampicilin
ARS	Autonomously Replicating Sequence
AS	antisense
bBBr	Dibromobimane
BglII	restriction enzyme
BMOE	Bismaleimidoethane
bp	base pair
BSA	bovine serum albumine
C, Cys	cysteine
CAD	CENP-A nucleosome distal complex
CATD	CENP-A targeting domain
CCAN	constitutive centromere associated complex
CDE	CEN DNA element
Cdk	cyclin dependent kinase
CEN	centromeric DNA
CENP-A	centromere protein A
ChIP	chromatin immunoprecipitation
CID	CENP-A homologue in fruit fly
CL	cross-link
CPC	chromosomal passenger complex
CREST	calcinosis, Raynaud's phenomenon, esophageal dysmotility, sclerodactyly, and telangiectasia
Cse4	CENP-A homologue in budding yeast
D, Asp	aspartate
dCTP	deoxycytidine triphosphate
DIG	digoxygenin

DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
Dnase	deoxyribonuclease
dNTP's	deoxyribonucleotides
DTNB	Ellman's reagent, 5,5'-dithiobis(2-nitrobenzoate)
DTT	dithiothreitol
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
fg	femtogram
FL	full-length
g	gram
h	hour
HA	hemagglutinin
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HFD	histone fold domain
HJURP	holliday junction recognition protein
HPH	hygromycin
HRP	horse radish peroxidase
I, Ile	isoleucin
IP	immunoprecipitation
K, Lys	lysine
KAN	kanamycin
kb	kilobase
kDa	kilodalton
l	liter
L, Leu	leucine
LB	Luria Broth
LD	loading dye
leu	leucine
LiAc	lithium acetate
LNA	locked nucleic acid
M	molarity g/mol
MAT	mating type

Mbp	megabasepairs
MDa	megadalton
mg	milligram
min	minute
ml	milliliter
MNase	micrococcal nuclease
MQ	milli Q
Myc	epitope tag originating from c-Myc
NAC	CENP-A nucleosome associated complex
NAT	antibiotic nourseothricin
ng	nanogram
noc	nocodazole
nt	nucleotide
o/n	overnight
OD_x	optical density at x nm
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PEG	polyethylene glycol
pg	picogram
PIC	protease inhibitor cocktail
PMSF	phenylmethylsulphonyl fluoride
PTM	post translational modifications
qPCR	quantitative polymerase chain reaction
RNA	ribonucleic acid
RNAi	RNA interference
RNase	ribonuclease
rpm	rounds per minute
RT	room temperature
s	second
S, Ser	serine
SAP	shrimp alkaline phosphatase
Scm3	suppressor of chromosome missegregation, budding yeast homologue of HJURP

SDS	sodium dodecylsulfate
SE	sense
SSC	saline-sodium citrate buffer
ssDNA	salmon sperm DNA
T100	zymolyase
TAE	Tris-acetate-EDTA buffer
TBE	Tris-borate-EDTA buffer
TE	Tris-EDTA buffer
Tris	tris(hydroxymethyl)aminomethane
trp	tryptophane
TX-100	triton X 100
UV	ultraviolet
V	volt
w/	with
w/o	without
YE	yeast extract
µl	microliter

SUMMARY

For cell division, the replicated genetic information of a cell needs to be accurately segregated to the emerging daughter cells. This is achieved by packaging the DNA into sister chromatids, which are separated by the poleward pulling force of microtubules. Microtubules are attached to the centromeres of the sister chromatids by a multi-protein complex called the kinetochore. The assembly of the kinetochore at the centromere is directed by specialized centromeric chromatin. Conventional chromatin contains nucleosomes, which are comprised of two copies each of the histones H3, H4, H2A, and H2B. This protein octamer organizes 147 bp of DNA by wrapping it in 1.7 turns. In contrast to canonical nucleosomes it is believed that centromeric nucleosomes are devoid of histone H3 and contain in its place the variant CENP-A. CENP-A is an essential protein necessary for kinetochore formation and homologues have been identified in all eukaryotes studied so far. Whereas higher eukaryotes have long arrays (kilo- to megabases of DNA) of centromeric chromatin with CENP-A containing nucleosomes, the budding yeast centromeric DNA is approximately 125 bp with only a single Cse4 (budding yeast homologue of CENP-A) containing nucleosome that is sufficient to recruit and assemble the kinetochore. This so-called point centromere is thought to represent the smallest unit of larger centromeres. However, the exact composition of this special centromeric nucleosome is subject of intensive debate with contradicting models proposed.

We investigated the exact composition of the centromeric nucleosome in budding yeast by developing a novel chromatin immunoprecipitation technique. This ChIP approach was based on the use of a centromeric DNA fragment that is too short to accommodate more than a single nucleosome. Not only did we observe the interaction of CENP-A^{Cse4}, histones H4, H2A, and H2B with the centromeric DNA, we also discovered a strong association of histone H3 with this fragment. By employing a sequential ChIP approach we could show that histone H3 and CENP-A^{Cse4} are co-occupying the centromeric DNA. Our experimental evidence supports the view of a heterotypic H3/CENP-A^{Cse4} nucleosome at the centromere and all future models need to account for the presence of histone H3 at the centromeric DNA.

ZUSAMMENFASSUNG

Für die Zellteilung muss die replizierte genetische Information einer Zelle korrekt auf die entstehenden Tochterzellen aufgeteilt werden. Die DNS wird dafür in Schwesterchromatiden verpackt, welche mit Hilfe von Mikrotubuli zu den entgegengesetzten Zellpolen gezogen werden. Die Mikrotubuli sind dafür mit den Zentromeren der Schwesterchromatiden über einen Multiproteinkomplex, dem Kinetochor, verbunden. Der Aufbau des Kinetochors findet gezielt an dem speziellen Chromatin des Zentromers statt. Kanonisches Chromatin besteht aus Nukleosomen, welche aus je zwei Molekülen der Histone H3, H4, H2A und H2B zusammengesetzt sind und diese Proteinoktamere sind von 147 bp DNS in 1.7 Windungen umwickelt. Im Vergleich dazu enthalten zentromere Nukleosomen die Histon H3 Variante CENP-A und es gibt die Auffassung, dass diese Nukleosomen kein Histon H3 beinhalten. CENP-A ist ein essentielles Protein, welches unerlässlich ist für den Aufbau des Kinetochors. Homologe Proteine von CENP-A wurden in allen untersuchten Eukaryoten entdeckt. Während höhere Eukaryoten lange Reihen (kbp bis Mbp DNS) von zentromeren Chromatin mit CENP-A Nukleosomen besitzen, hat die zentromere DNA der Sprosshefe nur eine länge von ungefähr 125 bp mit einem einzigen Cse4 (Sprosshefehomolog von CENP-A) beinhaltendem zentromeren Nukleosom. Dieses Nukleosom ist ausreichend um Kinetochorproteine zu rekrutieren und den Multiproteinkomplex aufzubauen. Bei diesem sogenannten Punktzentromer nimmt man an, dass es die kleinste Einheit von größeren Zentromeren darstellt. Allerdings wird der genaue Aufbau des speziellen zentromeren Nukleosoms mit verschiedenen, einander widersprechenden Modellen kontrovers diskutiert.

Wir haben eine neuartige Chromatinimmunopräzipitationstechnik (ChIP) entwickelt um den exakten Aufbau des zentromeren Nukleosoms in Sprosshefe zu untersuchen. Dieser neue ChIP-Ansatz basiert auf der Anwendung eines zentromeren DNS-Fragments, das zu kurz ist um mehr als einem Nukleosom Platz zu bieten. Unsere Ergebnisse bestätigten die Assoziation von CENP-A^{Cse4}, Histon H4, H2A und H2B mit der zentromeren DNS. Jedoch entdeckten wir auch eine starke Interaktion von Histon H3 mit diesem DNS-Fragment. Mit der Durchführung eines sequenziellen ChIP-Ansatzes

konnten wir zudem zeigen, dass Histon H3 und CENP-A^{Cse4} gleichzeitig mit der zentromeren DNS assoziiert sind. Unsere Ergebnisse legen nahe, dass es sich bei dem zentromeren Nuklesom in Sprosshefe um ein heterotypisches Nukleosom, mit je einem Molekül Histon H3 und CENP-A^{Cse4} handelt. Zukünftige Modelle müssen Histon H3 in den Aufbau des zentromeren Nukleosomes mit einbeziehen.

1 Introduction

1.1 How microtubules attach to chromosomes and mediate chromosomal segregation during cell division

The cell is the smallest unit of life. Cell division is the basis of growth and reproduction, which are fundamental qualities of life. The division of a cell is a process during which a mother cell splits into two daughter cells inheriting identical sets of genetic information stored in form of DNA. The DNA of the mother cell is replicated and then equally distributed between the two emerging daughter cells. The genome of a eukaryotic cell is divided into chromosomes. During cell division, a so-called primary constriction, more commonly referred to as a centromere, can be observed on the condensed chromosomes. The centromere is the region of the chromosome where the two sister chromatids are held most closely together due to the cohesion between them. The centromere divides the chromosome into left and right arms. The regions at the ends of the chromosome have a very special structure and are called telomeres. To segregate the sister chromatids to the daughter cells, the chromosomes align at the equator of the cell, and microtubules attach to the centromeres of sister chromatids. The microtubules are dynamically unstable polymers of alpha and beta tubulin that alternate between growing and shrinking. The minus ends of the microtubules with exposed alpha tubulin are located at either one of the two centrosomes localizing to the opposite poles of the cell (in yeast termed spindle pole bodies, or SPBs). The plus ends of beta tubulin are connected to the centromere. In addition to microtubules that connect spindle poles to centromeres, there are pole-to-pole microtubules and astral microtubules. Together they form the mitotic spindle, which controls the chromosome movement and segregation.

The attachment of the microtubule to the centromere is mediated by a protein complex called the kinetochore. The pole-to-kinetochore microtubule is referred to as k-microtubule. For proper inheritance of genetic information, it is crucial that only one kinetochore assembles per chromatid. The presence of two separate kinetochores on the same chromatid will result in chromosomal bridges. The chromatid might be physically torn apart by the spindle forces

leading to the loss or gain of genetic information. In order to properly segregate the sister chromatids to daughter cells, the sister kinetochores have to attach to microtubules that originate from the opposite poles. This arrangement is termed an amphitelic attachment or a bipolar orientation (Figure 1.1A). If the sister chromatids are attached to the microtubules in a bipolar fashion, then shortening of the k-microtubules will pull the sister chromatids in opposite directions, generating tension, and overcoming the cohesive forces between the sisters.

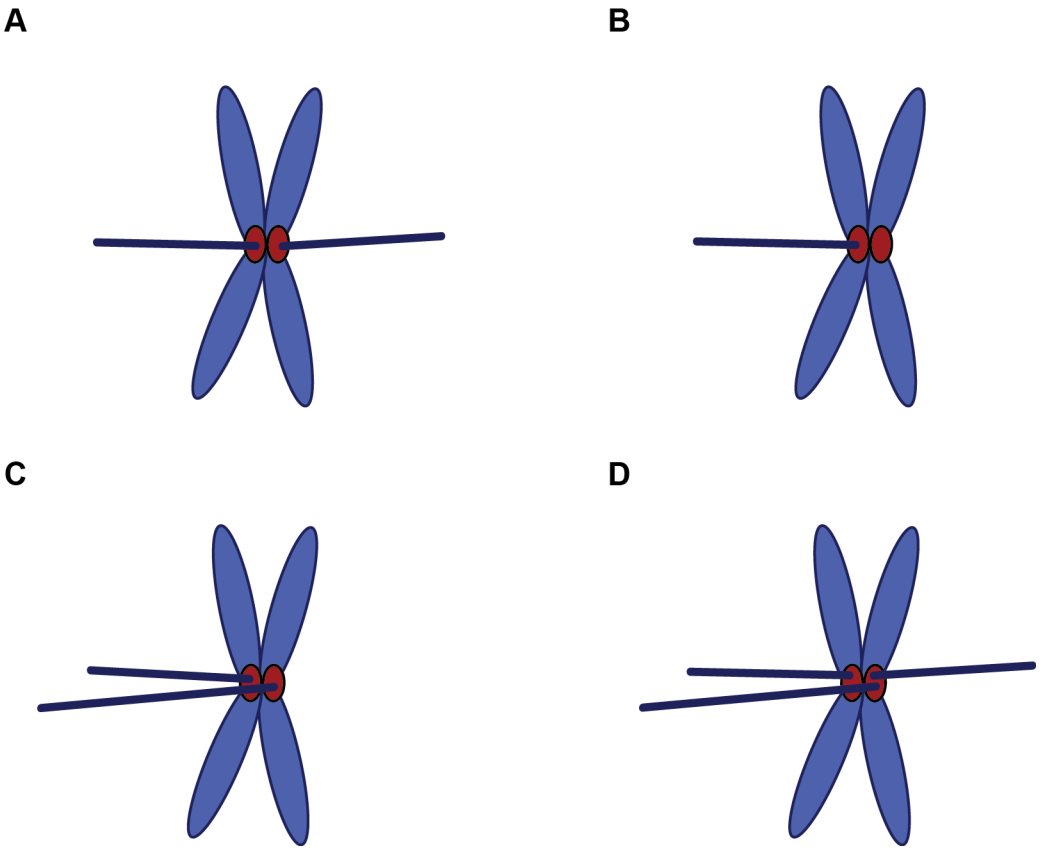

Figure 1.1 Modes of chromosome attachment
The chromosome is depicted in blue, sister kinetochores are depicted in red and the microtubules are shown in dark blue. A) Bipolar orientation or amphitelic attachment. The sister kinetochores are attached to microtubules that originate from opposite poles. B) Monotelic attachment. Only one of the two sister kinetochores is attached to microtubules. C) Syntelic attachment. Both kinetochores are attached to microtubules originating from the same pole. D) Merotelic attachment. One of the kinetochores is attached to microtubules from both poles.

In the case of a monotelic attachment (Figure 1.1B) only one of the two sister kinetochores is attached to microtubules of one of the two centrosomes. The situation where both sister kinetochores attach to microtubules from the

same centrosome is called syntelic attachment (Figure 1.1C). The monotelic and syntelic modes of attachment, if left uncorrected, will lead to non-disjunction of sister chromatids and aneuploidy. Missegregation can also happen when one sister kinetochore is attached to microtubules of both centrosomes at the same time, which is called merotelic attachment (Figure 1.1D). It is important to note that only amphitelically attached microtubules will generate tension and split the sister kinetochores. The attachment of the microtubule to the kinetochore is controlled by the spindle checkpoint, which can sense the tension that is created when the chromatids are properly attached to the spindle.

The kinetochore is a very large structure with a diameter of 200 - 250 nm in vertebrate cells. Even in budding yeast that attach just a single microtubule per chromosome, the kinetochore is comprised of more than 120 different proteins, which act in concert to orchestrate the accurate microtubule attachment, chromosome movement and the spindle checkpoint (De Wulf and Earnshaw, 2009). The budding yeast kinetochore is comprised of at least 17 sub complexes with a total mass exceeding 5 MDa (De Wulf et al., 2003). Early electron microscopy studies of vertebrate kinetochore were conducted by (Brinkley and Stubblefield, 1966) and (Jokelainen, 1967), who observed a disc-shaped three-layer structure and coined the terms for the inner, middle and outer kinetochore referring to the three layers (Jokelainen, 1967).

The outer plate of a vertebrate kinetochore contains about 20 anchoring sites for the plus ends of k-microtubules. The outer kinetochore includes stable constitutive components that mediate the direct interactions between k-microtubules, the kinetochore, and dynamic components, which are bound to the kinetochore only at certain stages of the cell cycle (De Wulf and Earnshaw, 2009).

In vertebrate cells, the centrosomes are located in the cytoplasm and the microtubules can attach to the kinetochores only after the nuclear envelope breaks down in prophase of mitosis. In budding yeast, spindle poles are incorporated in the nuclear envelope and kinetochores remain attached to the microtubules for most of the cell cycle (Kitagawa and Hieter, 2001). However, if the attachment is lost as a result of treatment with a microtubule-depolymerizing drug, kinetochores can efficiently re-attach to newly growing

microtubules. The microtubules of the spindle grow and shrink in various directions and 'search' for the kinetochores of chromosomes. In vertebrate cells, they are guided by a Ran-GTP protein gradient in the vicinity of chromosomes. The microtubules that originate from the kinetochore further facilitate kinetochore attachment to the spindle. Kinetochores first associate with the lateral surface of the microtubules. This so-called lateral attachment is more efficient due to the much larger contact surface (referred to as lattice) compared to the tip of a k-microtubule. The lateral attachment is later converted into an end-on attachment (Tanaka, 2010). Once a chromosome is captured by a microtubule it moves towards the spindle pole. This movement is promoted by the minus end directed motor protein dynein and the plus end directed motor kinesin CENP-E, which also moves in the direction of the centrosome due to the polarity conversion of the microtubules originating from the kinetochore. In addition, motor proteins promote spindle elongation by sliding microtubules along each other. Some of them can also depolymerize microtubules. Microtubule shrinkage from the plus end leads to the tethering of the kinetochore at this site. In amphitelic attachment, the second sister kinetochore needs to 'capture' k-microtubules from the opposite spindle pole. Amphitelic attachment is facilitated by the back-to-back position of the sister kinetochores. With the binding of the kinetochore to k-microtubules, the motor proteins are released from the kinetochore and attachment to the tubulin lattice is further mediated by the Ndc80 protein complex of the outer kinetochore (Tanaka, 2010). The latter is a rod shaped heterotetramer, which has two globular domains separated by an intermolecular coiled-coil. One of the globular domains attaches to the microtubule and the other is connected to the Mis12/KNL1 complex of the outer kinetochore. KNL1 can also contact the microtubules directly. KNL1, Mis12, Ndc80 protein complexes are referred to as the KMN network, which is connected to the inner kinetochore. The lateral attachment of the kinetochore to the microtubule can be mediated by the KMN network alone. However, for the stable connection of the kinetochore to the shrinking microtubule end the Dam1 complex (also called DASH) is required. This protein complex consists of ten components that form a ring around the dynamic microtubule end (Santaguida and Musacchio, 2009). It was shown that

Dam1, when artificially tethered to a minichromosome without a centromere, is sufficient to ensure its segregation during mitosis (Kiermaier et al., 2009; Lacefield et al., 2009). The Dam1 ring is connected to the KMN network via Ndc80.

A key factor in the correction of anchoring errors like syntelic attachment is the four subunit chromosomal passenger complex (CPC), which localizes to the center kinetochore and contains the protein kinase Aurora B. Aurora B destabilizes incorrect microtubule attachments by phosphorylating members of the Ndc80 and Dam1 complexes. It has been proposed that the localization of Aurora B in the inner region between the sister kinetochores is important for its function. When the sister kinetochores separate and tension is established, Aurora B cannot reach its substrates and microtubule attachment is stabilized (van der Waal et al., 2012).

1.2 The organization of the centromeric DNA is very divergent among species

What is the mechanism that ensures kinetochore assembly at the centromere? In budding yeast the key factor that determines kinetochore localization is the sequence of the centromeric DNA. The so-called point centromeres of budding yeast have a size of 111 to 119 bp and contain three centromeric DNA elements (Fitzgerald-Hayes et al., 1982; Hieter et al., 1985). CDEI is a conserved 8 bp palindrome, CDEII has a conserved length of 78 to 86 bp and contains about 90 % A/T, and CDEIII is 25 bp long with a highly conserved central region (Fleig et al., 1995; Hegemann and Fleig, 1993) (Figure 1.2). All 16 chromosomes of budding yeast contain this CEN DNA, which is interchangeable between different chromosomes (Clarke and Carbon, 1983). The orientation of the CEN DNA relative to the chromosome can be changed without consequence. However, the orientation of CDEIII with respect to CDEI and II is important. Deletion of CDEI alone leads to high chromosome loss, whereas the removal of CDEIII leads to the total loss of centromere function (Hegemann and Fleig, 1993). It was shown that 125 bp of CEN6 including CDEI-III is sufficient for centromere function when this sequence is introduced in a plasmid (Cottarel et al., 1989). The loss of the centromeric DNA leads to the

instability of the resulting acentric chromosome (Clarke and Carbon, 1983). A plasmid with two CEN DNA sequences is called dicentric and is also unstable due to the formation of two kinetochores and breakage of the plasmid (Mann and Davis, 1983). These early experiments showed that in budding yeast the underlying CEN DNA sequence is essential for the formation of a functional kinetochore and that centromeric DNA is recognized by specific factors, which initiate kinetochore assembly.

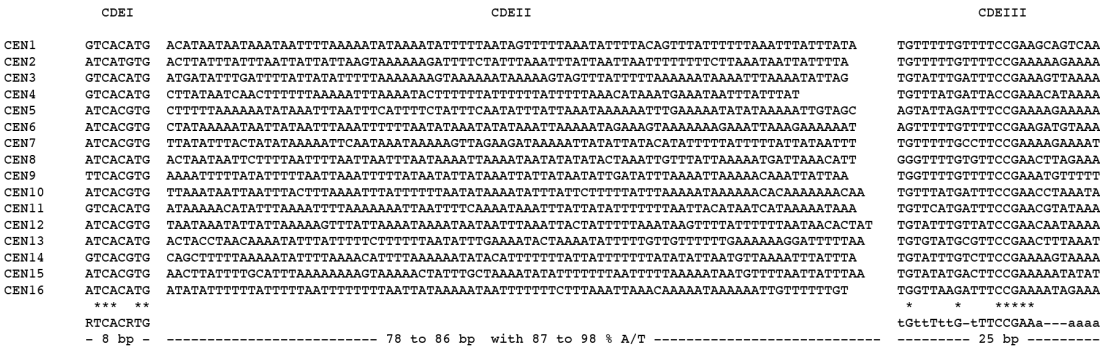

Figure 1.2 Alignment of the centromeric DNA sequences of S.cerevisiae
The CEN DNA sequences were retrieved from the Saccharomyces Genome Database (SGD, www.yeastgenome.org). The centromeric DNA consists of three centromeric DNA elements CDEI with 8 bp, CDEII with 78 to 86 bp and CDEIII with 25 bp. The asterisks below the alignment indicate the conservation of the DNA residues among all 16 centromeres. Bottom: The consensus sequence of CDEI and CDEIII are indicated in uppercase letters for highly conserved bases present in 15 of 16, CEN DNAs (R stands for purine base), conserved bases are shown in lowercase letters (10 to 14 of the 16 CEN DNAs), and non-conserved positions are indicated by dashes. Adapted from (Hegemann and Fleig, 1993).

In contrast to budding yeast, the centromeric DNA of fission yeast and higher eukaryotes is much longer and more complex. Their centromeres are referred to as regional centromeres. In the case of fission yeast, the centromeric DNA is 30 to 110 kb in size and is comprised of A/T rich DNA in the core with a size of 4 to 10 kb, which is surrounded by DNA repeats. The centromeric DNA in human cells varies size from 200 kb up to 4 Mb and consists of alpha satellite repeats or alphoid DNA with a length of 171 bp.

Are the longer regional centromeres comprised of multiple point centromeres? In the early 90's, an interesting observation led to the repeat 'subunit' model for the kinetochores of higher eukaryotes. Two closely related species of muntjac deer, the Indian and the Chinese muntjacs, are capable of interbreeding although they posses a very different the number of

chromosomes. The Chinese muntjac has 46 small chromosomes (2N), whereas the Indian muntjac male and female have 7 and 6 large chromosomes, respectively. This is the lowest chromosome number in a mammal described so far. The genomes of these two species are very similar and there is evidence that the 7 chromosomes of the Indian muntjac resulted from the fusion of the 46 chromosomes of the Chinese muntjac (Tsipouri et al., 2008). But if the chromosomes were fused, can they be now split apart again? Cells of the Indian muntjac were arrested in the G1 phase of the cell cycle and treated with hydroxyurea and caffeine to block the replication of DNA. This treatment caused the fragmentation of chromosomes and their kinetochores, which were detached from the underlying centromeric DNA. Instead of the original seven chromosomes, each with a pair of kinetochores, now 80-100 chromosomal fragments could be visualized by microscopy. These fragments were still attached to microtubules and could also become bi-oriented in the case of larger fragments. The authors also noticed a discontinuous staining of stretched centromeric DNA fibers with antibodies, which stain specific kinetochore proteins. They proposed that the kinetochore is assembled by the condensation of similar repetitive modules consisting of a microtubule binding segment and a linker segment in a solenoid fashion with the microtubule segment pointing poleward (Brinkley et al., 1992; Zinkowski et al., 1991). One implication of this model is that the basic kinetochore of budding yeast represents a functional microtubule-binding module.

Unlike the budding yeast point centromere, the underlying DNA sequence in regional centromeres is not conserved. Closely related species have virtually no sequence similarity in their centromeres, and the length of the centromeric DNA is very different even between different chromosomes in one cell. Deletion of a regional centromere leads to the formation of functional kinetochores at an ectopic locus of the chromosome. The newly formed centromere is called neocentromere and the underlying DNA has no sequence similarity to the original centromeric DNA (Ishii et al., 2008). Surprisingly, the addition of a second regional centromeric DNA sequence to a chromosome does not result in the formation of a second centromere and chromosome bridges. The chromosome assembles a kinetochore only at one of the centromeres, and

the second centromere remains inactive (Agudo et al., 2000; Earnshaw and Migeon, 1985; Sullivan and Willard, 1998).

1.3 The epigenetic inheritance of the centromere

If the DNA sequence does not define the site of kinetochore assembly in higher eukaryotes, what does? The DNA in a cell is not naked; it is complexed with protein and known as chromatin. In fact, the amount of protein and DNA is nearly similar in chromatin. The basic unit of chromatin is the nucleosome, which consists of a protein complex with DNA wrapped around it. The presence of nucleosomes was first deduced from biochemical experiments in which chromatin was digested with a nuclease that preferentially cuts the linker region of ~60 bp between nucleosomes. A characteristic digestion pattern with the smallest DNA fragment of ~150 bp was observed, indicating the presence of evenly spaced protein complexes protecting the DNA from nucleases. This 'beads on a string' organization could also be visualized by electron microscopy (Rattner and Hamkalo, 1978). The canonical nucleosome is an octamer comprised of two copies each of histones H3, H4, H2A, and H2B. Histones are highly conserved proteins among eukaryotes. They are characterized by the histone fold domain (HFD), which consists of three alpha helices separated by loops (α1-L1-α2-L2-α3). Histones H3 and H4 form a histone-fold dimer in a head-to-tail fashion, which is referred to as a "handshake" motif. Two of those H3-H4 dimers build a hetero-tetramer via a four-helix bundle that is comprised of the α2 and α3 helices of the two H3 molecules. The H3-H4 heterotetramer is complemented by two histone-fold dimers, consisting of H2A and H2B, via homologous four-helix bundles formed between H2B and H4. The histone octamer displays a two-fold symmetry around a dyad axis that passes through the H3-H3' four-helix bundle dimerization interface. 147 bp of DNA are organized with 1.65 turns around the histone octamer in a flat left-handed superhelix starting at H3 and ending at H3'. The start and end points of the DNA histone interaction are also referred to as the entry and exit sites. More than 140 hydrogen bonds stabilize the interaction between DNA and protein at the nucleosome (Luger et al., 1997) (Figure 1.3).

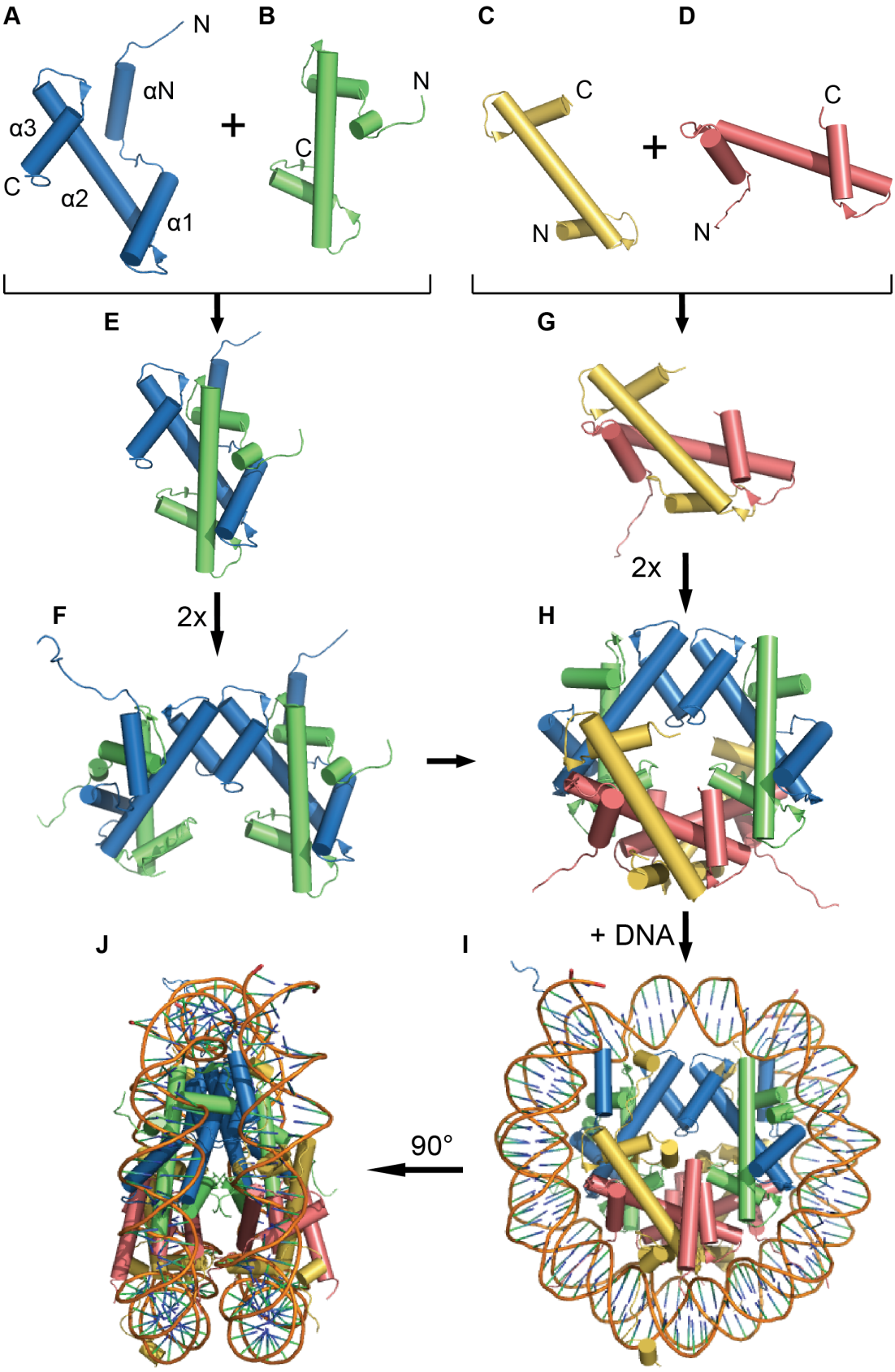

Figure 1.3 The nucleosome assembly

Histone H3 is shown in blue (A), with αN, α1, α2, and α3 indicated, histone H4 in green (B), histone H2A in yellow (C), and histone H2B (D) in red. N indicates the N-terminus, C stands for C-terminus. E)

The histone H3/H4 heterodimer is formed by the head-to-tail dimerization of histones H3 and H4. F) The histone $(H3/H4)_2$ heterotetramer is formed by the dimerization of two histone H3/H4 heterodimers via a four-helix bundle built of the α2 and α3 helices of histones H3. G) The H2A/H2B heterotetramer is formed by the head-to-tail dimerization of histones H2A and H2B. H) The histone $(H3/H4/H2A/H2B)_2$ octamer is formed by the assembly of the $(H3/H4)_2$ heterotetramer with two H2A/H2B heterodimers. Only the histone folds are shown. I) The canonical nucleosome. 147 bp of DNA are organized by the histone octamer in a lefthanded manner, the dyad axis of the nucleosome passes through the H3-H3' four-helix bundle dimerization interface. J) The side view of (I). The structure was published in (Luger et al., 1997). Adapted from (Alberts, 2002).

The high-resolution crystal structure of the nucleosome that was solved in 1997 lacks information about the N- and C-terminal tails of histones. Histone tails are very flexible and are thought to be responsible for the higher order structure of chromatin. An array of nucleosomes folds into a 30 nm fiber, in which the histone tails are thought to interact with neighboring nucleosomes (Horn and Peterson, 2002). The N-terminal tails of histones are post-translationally modified. Each tail is subject to several types of covalent modifications including the acetylation and methylation of lysines, and serine phosphorylation. Histone modifications can carry information regarding the functional state of the DNA that is associated with the modified nucleosomes and provide an epigenetic memory. Gene transcription can be regulated without changing the underlying DNA sequence, and thereby different cell types can develop in a multicellular organism subject to differential modification of histones in the promoter regions of cell type-specific genes.

Another level of chromatin regulation is provided by histone variants. There are two types of histone isoforms; The homomorphous variants, which differ from the canonical histones only by a few amino acids, and the more divergent heteromorphous variants. Histone variants play an important role in development, chromosome segregation, double-strand break DNA repair, recombination, replication, spermatogenesis, and X chromosome inactivation (Ausio, 2006). Some histone variants are specifically located in silenced heterochromatin, actively transcribed euchromatin, or centromeric chromatin.

The centromeric DNA of all eukaryotes studied so far is marked by a special histone variant called CENP-A (Centromeric Protein A). This protein was first identified with centromere-specific autoimmune antisera from patients with CREST syndrome (calcinosis, Raynaud's phenomenon, esophageal

dysmotility, sclerodactyly, and telangiectasia) (Compton et al., 1991; Earnshaw and Rothfield, 1985; Earnshaw and Tomkiel, 1992; Palmer and Margolis, 1985). CENP-A was demonstrated to be a histone H3 variant with more than 60 % similarity between the two (Palmer et al., 1991; Palmer et al., 1987; Stoler et al., 1995; Sullivan et al., 1994) (Figure 1.4). These homologues are well conserved from fungi to humans (Figure 1.5). In budding yeast, the CENP-A homologue Cse4 (thereafter referred to as CENP-A^{Cse4}) was discovered in a screen for mutants defective in mitotic chromosomal segregation (Stoler et al., 1995). Its similarity to histone H3 is limited to the histone fold domain (HFD) and an extra N-terminal alpha helix. The N-terminal tails of CENP-A homologues from different organisms are highly divergent in length and amino acid sequence (Henikoff and Dalal, 2005; Malik and Henikoff, 2009) (Figure 1.6). CENP-A has been proposed to replace histone H3 in a specialized centromeric nucleosome, and thereby mark the centromere for the assembly of 'downstream' kinetochore proteins.

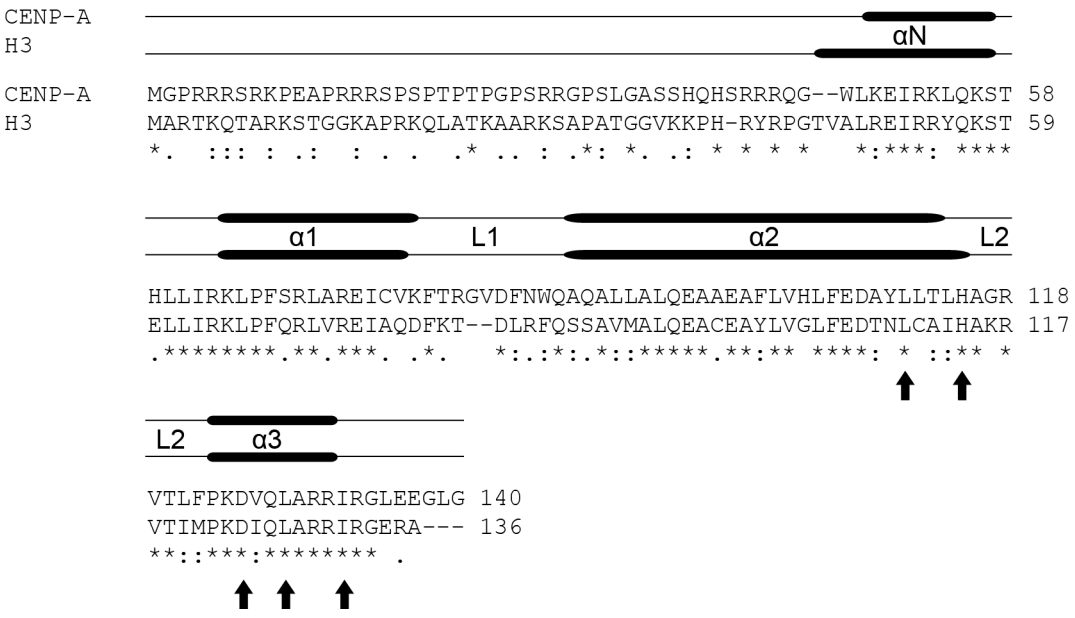

Figure 1.4 Clustal W alignment of human CENP-A with histone H3

At the top, the secondary structures of CENP-A and H3 are indicated: α is an alpha helix and L indicates a loop. The histone fold domain is comprised of the α1-L1-α2-L2-α3. The asterisk (*) indicates fully conserved residues, the colon (:) indicates residues with similar properties (e.g. hydrophobic residues L and I) and the dot (.) indicates residues with weakly similar properties (e.g. charged residues E and R). The black arrows highlight residues, which are important for the dimerization of H3 and CENP-A via the four-helix bundle comprised of the α2 and α3 helix.

1.4 The role of CENP-A in the kinetochore assembly.

The recruitment of CENP-A to the centromeric DNA is a pre-requisite for the assembly of the kinetochore. The recruitment of Drosophila CENP-A^{CID} -LacI fusion to an ectopic locus on a chromosome results in the recruitment of the components of the inner kinetochore, the KMN network, and the formation of a functional kinetochore (Mendiburo et al., 2011).

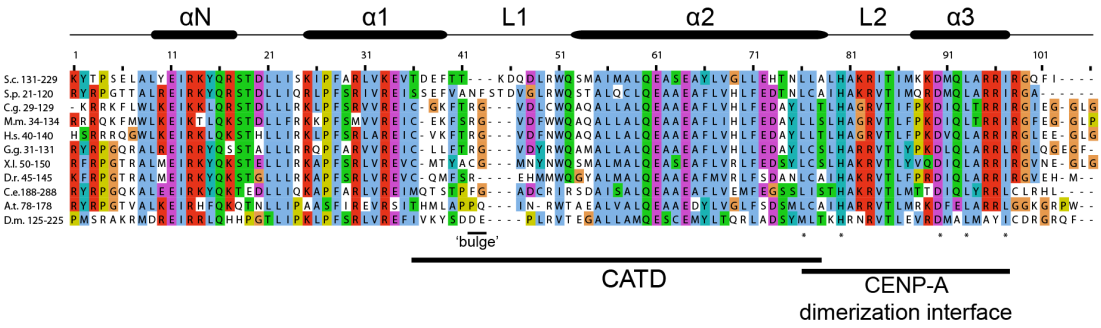

Figure 1.5 CENP-A homologues are a highly conserved among species
Shown is the ClustalW (www.ebi.ac.uk/Tools/msa/clustalw2/) alignment of the C-terminal ~100 amino acid of CENP-A from different species (*Saccharomyces cerevisiae* (S.c.), *Schizosaccharomyces pombe* (S.p.), *Cricetulus griseus* (C.g.), *Mus musculus* (M.m.), *Homo sapiens* (H.s.), *Gallus gallus* (G.g.), *Xenopus laevis* (X.l.), *Danio rerio* (D.r.), *Caenorhabditis elegans* (C.e.), *Arabidosis thaliana* (A.t.), and *Drosophila melanogaster* (D.m.)) prepared with JalView 2.6.1. and the clustalX color setting. The histone fold domain is comprised of the α1-α2-α3 helices. Below the alignment, the asterisks indicate residues important for the homodimerization of CENP-A in the nucleosome self-association interface, and CATD stands for CENP-A targeting domain (see chapter 1.8 and 1.13 for further information).

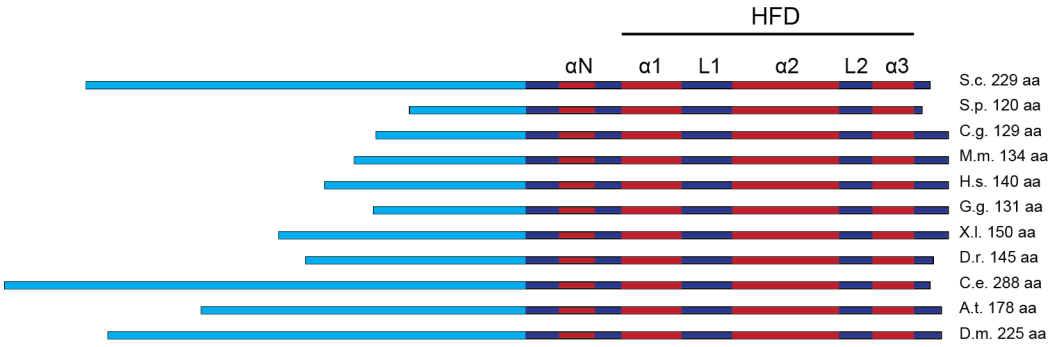

Figure 1.6 The N-termini of CENP-A from different species are very diverged in length
Shown are schemes of CENP-A homologues from *Saccharomyces cerevisiae* (S.c.), *Schizosaccharomyces pombe* (S.p.), *Cricetulus griseus* (C.g.), *Mus musculus* (M.m.), *Homo sapiens* (H.s.), *Gallus gallus* (G.g.), *Xenopus laevis* (X.l.), *Danio rerio* (D.r.), *Caenorhabditis elegans* (C.e.), *Arabidosis thaliana* (A.t.), and *Drosophila melanogaster* (D.m.). The N-terminal regions are depicted in light blue, the secondary structure (α helices in red, loops in dark blue) and the HFD are indicated in the figure.

Some of the kinetochore components interact with CENP-A directly and could be co-immunoprecipitated with CENP-A in human cells. These components are defined as inner kinetochore and were named the CENP-A nucleosome associated complex (NAC). Several NAC components contain DNA-binding motifs and are capable of interacting with DNA directly. An additional class of kinetochore proteins, the CENP-A nucleosome distal complex (CAD), co-immunoprecipitate with components of the NAC but not with CENP-A. Since a number of proteins were identified simultaneously as the members of both the NAC and the CAD complexes, the NAC/CAD complex was re-named constitutive centromere associated network (CCAN). In humans, 16 components of the CCAN have been identified to date. The bridging of the CCAN to the plus end of the microtubule is mediated by the KMN network. Almost all CCAN and KMN components have homologues in budding yeast (Kitagawa and Hieter, 2001). The hierarchical order of assembly of the CCAN and the KMN networks was determined by localization dependencies in different knockout cell lines and by the tethering of certain components to non-centromeric regions (Perpelescu and Fukagawa, 2011).

1.5 The C-terminus of CENP-A and its connection to the CCAN network

The six amino acids at the extreme C-terminus of CENP-A immediately following alpha helix 3 are conserved between mammals (human, mouse, and hamster) and amphibians (Xenopus) (Figure 1.5). In Xenopus, the C-terminus of CENP-A is crucial for the recruitment of CENP-C, a component of the CCAN network (Carroll et al., 2010; Guse et al., 2011). In recent studies, a histone H3-CENP-A chimera was assembled into nucleosomes on DNA beads. When the beads were incubated with Xenopus egg extracts, they recruited CENP-C and assembled the functional kinetochore, but only if the chimera contained the C-terminal amino acids of CENP-A. On the contrary, in human cells an H3/CENP-A chimera with the C-terminus of H3 recruits CENP-C to the centromeres in vivo (Black et al., 2007). It is possible that multiple pathways are responsible for CENP-C centromeric localization in live cells, and only some of them are functional in egg extracts. Alternatively, the requirement for the CENP-A C-

terminus may be different in humans and frogs.

The CENP-A C-terminus is highly divergent among fruit fly, zebra fish, budding and fission yeast, and arabidopsis. In budding yeast, CENP-A^{Cse4} was shown to co-purify with the CENP-C homologue called Mif2 (Meluh and Koshland, 1995; Meluh and Koshland, 1997). However, it remains unknown whether the interaction is direct and by which part of CENP-A^{Cse4} it is mediated (Westermann et al., 2003).

1.6 The histone variants CENP-T-W-S-X and how they are embedded in the CCAN

Recently, four human kinetochore CCAN proteins structurally similar to canonical histones, CENP-T, CENP-W, CENP-S, and CENP-X, were described (Amano et al., 2009; Hori et al., 2008). Each of these proteins contains a histone-fold domain and heterodimers are formed between CENP-T/CENP-W and CENP-S/CENP-X, respectively. An asymmetric CENP-T-W-S-X heterotetramer, structurally similar to the $(H3/H4)_2$ heterotetramer, can be assembled from the CENP-T/CENP-W and CENP-S/CENP-X dimers via interactions between CENP-T and CENP-S. The CENP-T-W-S-X complex binds to DNA, protects ~100 bp in a nuclease digest, and can introduce supercoils into DNA (Nishino et al., 2012). Both CENP-S and CENP-X require CENP-T for their localization to the centromere (Amano et al., 2009), whereas CENP-T depends on CENP-A for its recruitment (Hori et al., 2008). However, there is no direct interaction between CENP-A and CENP-T (Hori et al., 2008). A very interesting feature of this new type of 'nucleosome' at the centromere is its connection to the KMN network via the extended N-terminus of CENP-T, which interacts directly with the Ndc80 complex and might form an additional scaffold that connects the centromeric DNA to microtubules. Another connection between the centromeric DNA and the Ndc80 complex is formed via CENP-C, which interacts with CENP-A and connects the centromeric nucleosome to the Ndc80 complex through Mis12. However, CENP-C and the components of the CENP-T-W-S-X complex do not interact with each other, and the recruitment of the CENP-T-W-S-X complex is independent of CENP-C (Amano et al., 2009; Hori et al., 2008).

The recent discovery of budding yeast homologues of CENP-T^{Cnn1}, CENP-W^{Wip1}, CENP-S^{Mhf1}, and CENP-X^{Mhf2} reveals that kinetochores that assemble on point and regional centromeres are in fact more similar than previously thought. In contrast to human, the budding yeast CENP-T-W-S-X complex is not essential. CENP-T^{Cnn1} was shown to interact with the Nd80 complex as it was observed in human. However, in budding yeast the interaction between CENP-T^{Cnn1} and the Ndc80 complex, and the interaction between the Ndc80 complex and Mis12/Mtw1 (Mis12^{Mtw1}) appear to be mutually exclusive (Bock et al., 2012; Schleiffer et al., 2012). It was proposed that there are about four times more Ndc80 complexes than CENP-A^{Cse4} molecules at the centromere (Coffman et al., 2011; Joglekar et al., 2006; Lawrimore et al., 2011). It is possible that several Ndc80 complexes are connected to the centromeric DNA via CENP-A^{Cse4}, whereas other Ndc80 complexes are associated with the DNA via the CENP-T-W-S-X complex.

The discovery of the CENP-T-W-S-X complex, which directly connects the centromeric DNA to the KMN network in budding yeast, provides new insights into the organization of a single microtubule-binding unit. A nucleosome has a diameter of ~10 nm, whereas a microtubule is ~25 nm in diameter. Two models of a microtubule-binding unit were proposed. In the vertical model, the "connector" proteins bound to the microtubule converge on a single nucleosome. In this case the pulling force of the microtubule would have to be resisted by a single centromeric nucleosome so that it does not get torn away from the centromeric DNA. (Santaguida and Musacchio, 2009). In the so-called horizontal model, the components of the KMN network are anchoring the microtubule to the neighboring H3 nucleosomes. In this model, the generated force is distributed over several contact points (Santaguida and Musacchio, 2009). Instead of the conventional H3 nucleosomes, the link between the peri-centromeric DNA and the KMN network might be provided by the CENP-T-W-S-X complexes. It is possible that both the vertical and the horizontal layouts simultaneously ensure the accurate attachment of the microtubule to the kinetochore.

1.7 The N-terminal tail is highly divergent among CENP-A homologues

In contrast to the conserved HFD of CENP-A, the N-terminal tails are very diverse in different organisms. The histone tail of budding yeast CENP-A^{Cse4} is ~130 aa long compared to ~40 aa for human CENP-A (Figure 1.7). A study by (Keith et al., 1999) revealed that the N-terminus of CENP-A^{Cse4} is essential. However, a shortened version consisting of the essential N-terminal domain (END domain, amino acid 28-60) fused directly to the HFD, restores the wild type function of CENP-A^{Cse4}, and is very similar in length to its human counterpart (Chen et al., 2000).

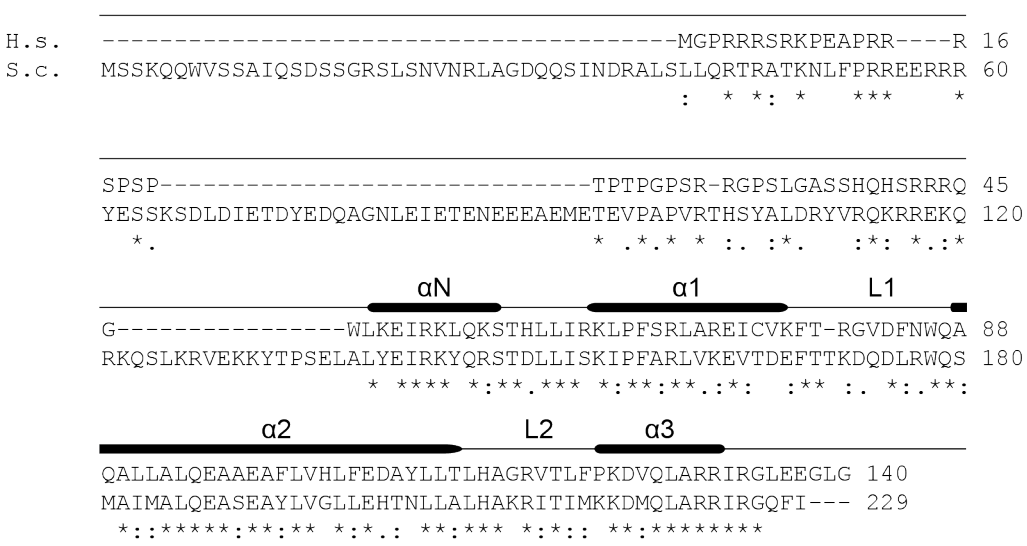

Figure 1.7 Alignment of CENP-A homologues from human and yeast
Shown is the ClustalW alignment of human CENP-A (H.s.) and budding yeast CENP-A^{Cse4} (S.c.). The secondary structure is indicated on the top. On the bottom of the alignment the asterisk (*) indicates fully conserved residues, the colon (:) indicates residues with similar properties and the dot (.) indicates residues with weakly similar properties.

Despite the differences between CENP-A homologues in different organisms, budding yeast and worm versions of GFP-CENP-A localize to centromeres when produced in human cells (Henikoff et al., 2000; Wieland et al., 2004). Furthermore, a block-of-proliferation resulting from the depletion of endogenous CENP-A by RNA interference (RNAi) in human cells was rescued by budding yeast CENP-A^{Cse4} expression (Wieland et al., 2004). Conversely, a HA-tagged CENP-A^{Cse4} construct localized to the nucleus in mammalian cells but failed to be recruited to the centromere. The attempt to rescue the lethality of a

CENP-A^{Cse4} deletion by expressing human CENP-A in budding yeast was also unsuccessful (Stoler et al., 1995). Since the N-terminus of CENP-A^{Cse4} is very different from the N-terminus of human CENP-A, it is possible that the human CENP-A protein lacks the motif(s) that are essential in budding yeast.

The histone tail of CENP-A^{Cse4} contains several sites that could be subject to post-translational modifications. However, no acetylation or phosphorylation of CENP-A^{Cse4} was reported to occur in budding yeast in vivo. Moreover, PTMs of the END domain are apparently not required for CENP-A^{Cse4} function since all modifiable residues could be mutated without causing an obvious defect (Chen et al., 2000). A CENP-A^{Cse4} mutant with all lysines in the entire protein sequence mutated to arginine could rescue a CENP-A^{Cse4} deletion strain (Collins et al., 2004). Recently, it was shown that CENP-A^{Cse4} is mono- and dimethylated on arginine 37 (R37). Although a budding yeast strain with a mutation of CENP-A^{Cse4} R37 to alanine (R37A) did not exhibit any growth defect, in combination with the deletion of the budding yeast-specific gene encoding the kinetochore protein Cbf1, the R37A mutation resulted in defective plasmid and chromosomal segregation (Samel et al., 2012).

In the human CENP-A homologue, none of the PTM sites found in the N-terminal tail of histone H3, namely phosphorylated S10 and acetylated K4, K14, K18 and K23 are present. The only posttranslational modification of CENP-A reported so far is S7 phosporylation by Aurora B kinase. The mutation of S7 to alanine or glutamine displayed a dominant negative effect over the endogenous wild type CENP-A and resulted in a delayed cytokinesis (Zeitlin et al., 2001). The S7 residue is not conserved in other species of mammals. However, all N-terminal tails of CENP-A homologues contain serine and/or threonine residues that could be potentially phosphorylated.

1.8 The CENP-A targeting 'domain' is necessary and sufficient for centromere localization

The amino acids required for the centromeric localization of CENP-A are located in the central portion of the histone fold domain. In human CENP-A, the histone fold domain spans 68 amino acids from K64 until I132. A chimeric protein with the N-terminal third of CENP-A (aa 1-51) and the C-terminal two

thirds of H3 (aa 52-135) fails to localize to the centromere, whereas the centromeric localization of a reciprocal chimeric protein with the N-terminal part from H3 (aa 1-53) and the C-terminal part from CENP-A (aa 54-140) is not impaired (Sullivan et al., 1994). The CENP-A targeting domain (CATD) was characterized more precisely by substituting CENP-A HFD amino acids for the residues found in H3. The residues responsible for centromeric localization map to the loop1 and the alpha2 helix (in human CENP-A amino acid 75 to 114)(Black et al., 2004; Shelby et al., 1997) (Figure 1.5). Replacing the corresponding region of H3 with the CATD of CENP-A in human cells targets the chimeric histone H3 to the centromere (Black et al., 2004). Furthermore, histone H3/CATD chimera can rescue the deletion of CENP-A. In cells expressing the chimeric protein all kinetochore components are recruited normally to the centromere and chromosomal segregation is normal demonstrating that the CATD is necessary and sufficient for the function of CENP-A (Black et al., 2007). A similar CATD was identified in budding yeast CENP-A^{Cse4}. However, in this organism the histone H3 CATD chimera can rescue the *cse4* deletion only if it includes the long N-terminal tail (aa 1-129) of CENP-A^{Cse4}, a substitution of lysine 126 for a glutamine in the alpha helix 3, and a change of three amino acid at the extreme C-terminus of H3 for those of CENP-A^{Cse4} (Black et al., 2007).

The mechanistic insights regarding the requirement of the CATD for CENP-A function were obtained only very recently. It was initially observed that the CATD results in a more rigid interface with H4 in the CENP-A/H4 heterotetramer compared to the H3/H4 heterotetramer (Black et al., 2004). However, the functional importance of this rigidity remains unknown. More revealing was the finding that the CATD is recognized by a histone chaperone Scm3.

1.9 CENP-A is recruited to the centromere by a chaperone

The assembly and disassembly of nucleosomes is facilitated by histone chaperones. The de novo assembly of nucleosomes is especially important after the replication of DNA. Histone chaperones bind to histone H3/H4 or H2A/H2B

dimers to prevent their aggregation. Nucleosomes are dynamic complexes, which are disassembled or dislodged during processes like DNA replication, gene expression, or DNA repair. Chromatin remodeling is assisted by histone chaperones. There are specialized histone chaperones with separate functions and with preferences for either H3/H4 or H2A/H2B dimers. For example, the histone chaperones ASF1 and CAF1 are important during DNA replication and repair and interact with the H3/H4 dimer. NAP1 is a histone chaperone for the H2A/H2B dimer that facilitates histone H2A variant exchange and support the process of nucleosomal sliding (Eitoku et al., 2008).

A recently discovered protein called Scm3 (suppressor of chromosome missegregation) in yeast (Camahort et al., 2007; Mizuguchi et al., 2007; Stoler et al., 2007) and HJURP (holliday junction recognition protein) in vertebrates (Dunleavy et al., 2009; Foltz et al., 2009) facilitates the assembly of CENP-A into the centromeric nucleosomes. The homology between HJURP and Scm3 is limited to 52 amino acids in the N-terminal part of HJURP (Sanchez-Pulido et al., 2009). A fragment of HJURP that only contains the Scm3 homology domain interacts with CENP-A, but it is unable to localize to the centromere in vivo (Shuaib et al., 2010). In vitro this fragment is sufficient to assemble CENP-A nucleosomes (Barnhart et al., 2011). When an HJURP-Lac repressor (LacI) fusion protein is artificially targeted to a Lac operator (Lac-O) array at an ectopic locus of the chromosome, CENP-A is recruited as well and persists at this locus even after the removal of HJURP-LacI. The HJURP Scm3-homologus fragment is sufficient to recruit CENP-A in this assay (Barnhart et al., 2011).

Last year, the structure of HJURP (Scm3) in complex with CENP-A and H4 was reported by three different laboratories (Cho and Harrison, 2011; Hu et al., 2011; Zhou et al., 2011). HJURP/Scm3 interacts with the CATD of CENP-A (Bassett et al., 2012). The binding of Scm3 is incompatible with the interaction between two CENP-A molecules within a $(CENP\text{-}A/H4)_2$ heterotetramer. Moreover, Scm3 binding to CENP-A would prevent CENP-A from interacting with the DNA. This finding is in contrast with an earlier report, which claimed that Scm3 is an integral component of a specialized CENP-A nucleosome where it substitutes for H2A/H2B dimers (Mizuguchi et al., 2007). In the view of the most recent studies, it appears that Scm3 association with CENP-A is only

transient and limited to CENP-A recruitment to the centromere. The role of Scm3 as a CENP-A chaperone is further supported by the observation that the co-expression of Scm3 with CENP-A^{Cse4} and H4 in bacteria reduces the formation of insoluble aggregates (Cho and Harrison, 2011).

In budding yeast, the centromeric localization of Scm3 is dependent on the recruitment of Ndc10 (Camahort et al., 2007). Ndc10 binds to CDEIII region of the centromere as a component of the four-protein CBF3 complex (Lechner and Carbon, 1991). The CBF3 complex is only found in species with a CDEIII motif. In addition, recombinant Ndc10 was reported to bind to CDEII DNA in vitro (Espelin et al., 2003). Ndc10 and Scm3 bind to each other, are recruited to the centromere in a mutually dependent manner (Camahort et al., 2007), and are both necessary for the localization of CENP-A (Camahort et al., 2007; Goh and Kilmartin, 1993). However, in contrast to Scm3, Ndc10 does not interact with CENP-A directly (Camahort et al., 2007). The recently solved crystal structure of Ndc10 and in vitro binding assays showed that Ndc10 homodimerizes and that Scm3 interacts with Ndc10 through a region, which is distinct from the CENP-A interacting domain. While the lethality of *scm3* deletion can be rescued by CENP-A^{Cse4} overexpression (Camahort et al., 2009), Ndc10 is an absolutely essential protein. It is worth mentioning that apart from its interaction with CBF3 and Scm3, Ndc10 plays a bigger role in kinetochore assembly and has additional binding partners within the kinetochore. It is interacting with a non-essential protein Cbf1 which, specifically recognizes CDEI region of the centromere (Niedenthal et al., 1991). A similar protein called CENP-B is found in human and binds to a specific sequence in alpha satellite DNA (Masumoto et al., 1989). Another interaction partner of Ndc10 is Bir1 the budding yeast homologue of survivin, which is involved in spindle attachment checkpoint and is a component of the chromosomal passenger complex (Cho and Harrison, 2012). Since the CBF3 complex can only be found in yeast with point centromeres it was speculated that in fungi with regional centromeres a C-terminal part of Scm3 performs CBF3 function and binds to the centromeric DNA (Aravind et al., 2007).

In fission yeast, CENP-A deposition at the centromere is dependent on the Mis16-Mis18 complex and Mis18 was shown to directly interact with Scm3

(Pidoux et al., 2009). However, in humans Mis18 α/β and HJURP could not be shown to associate with each other and the mechanism of how HJURP is recruited to the centromere remains unclear. There is some evidence that histone modification might play an important role. In higher eukaryotes, Mis16 (RbAp46) was found associated with histone acetyltransferases and it was proposed that histone acetylation might prime the centromere epigenetically for CENP-A deposition (Perpelescu and Fukagawa, 2011). Demethylation of histone H3 lysine 4 impairs the targeting of HJURP and CENP-A to centromeric chromatin of human artificial chromosomes (HAC) (Bergmann et al., 2011). The tethering of Suv39h1 methyltransferase that methylates K9 of histone H3, to an alpha satellite DNA sequence also inhibits CENP-A assembly (Ohzeki et al., 2012). It appears that the assembly of centromeric chromatin is facilitated by a certain pattern of histone modifications in the surrounding chromatin regions.

1.10 Regional centromeres exhibit specific histone modifications and are embedded into heterochromatin

In human centromeric DNA repeats, CENP-A nucleosomes are interspersed with canonical H3 nucleosomes (Blower et al., 2002). The whole centromeric region is embedded into heterochromatin. The post-translational modifications of canonical H3 nucleosomes in the centromeric chromatin are a unique combination of the modifications found in euchromatin and the flanking heterochromatin. The histone H3 in the flanking heterochromatin is di- and tri-methylated at K9. These modifications are absent in centromeric chromatin. However, the hypoacetylation of histone H3 and H4 is a common feature between the centromeric chromatin and the flanking heterochromatin (Sullivan and Karpen, 2004). On the other hand, K4 of histone H3 is di-methylated in the centromeric chromatin as observed in euchromatin. Moreover, the mammalian centromeric chromatin and the flanking heterochromatin was found to be associated with the histone variant H2A.Z, which replaces H2A in nucleosomes with histone H3 dimethylated on either K4 or K9 (Greaves et al., 2007). H2A.Z containing nucleosomes were reported to bind DNA more tightly than canonical nucleosomes (Kumar and Wigge, 2010).

In *D.melanogaster*, the chromatin of the centromeric DNA exhibits a very similar histone modification pattern as in human with regions of CENP-A chromatin interspersed with regions of H3 chromatin (Sullivan and Karpen, 2004). The deletion of flanking heterochromatic regions of the centromere in *Drosophila* results in the spreading of the centromeric chromatin and neocentromere formation (Maggert and Karpen, 2001).

In fission yeast, the kinetochore assembles at the central core region of the centromeric DNA consisting of non-repetitive DNA (*cnt*) and flanking inverted repeats called innermost repeats (*imr*). The cnt DNA is packaged into a unique chromatin structure, which upon limited micrococcal nuclease digest results in a smear rather than the canonical nucleosomal ladder suggesting a less regular spacing of nucleosomes (Marschall and Clarke, 1995). The central core is surrounded by silenced heterochromatin of the outer repeats (*otr*) (Pidoux and Allshire, 2005). Similarly to higher eukaryotes, the *otr* heterochromatin is hypoacetylated and histone H3 is methylated at K9 by the methyltransferase Clr4, which is a homologue of the Suv39h1 methyltransferase (Ekwall et al., 1995). The deletion of the centromere in fission yeast, leads to the formation of neocentromeres at subtelomeric regions, which have no sequence homology to the centromeric DNA, but they are in close proximity to the heterochromatic telomeric DNA (Buscaino et al., 2010; Ishii et al., 2008). Heterochromatin appears to be important for the assembly of kinetochores. One possible function of the flanking heterochromatin might be the delineation of the centromeric boundary. It was also hypothesized that heterochromatin provides the structural rigidity clamping the sites of microtubule attachment together. Chromosomes lacking peri-centromeric heterochromatin, display elevated rates of merotelic attachment (Gregan et al., 2007).

1.11 CENP-A abundance in the cell is tightly regulated

In higher eukaryotes, the overexpression of CENP-A causes its localization to ectopic sites on the chromosome, where it can direct the assembly of functional kinetochores resulting in the missegregation of chromosomes (Heun et al., 2006; Tomonaga et al., 2003; Van Hooser et al.,

2001). In budding yeast, the overexpression of CENP-A^{Cse4} does not lead to its localization to non-centromeric DNA, since CENP-A^{Cse4} has a very short half-life. However, the expression of a stable CENP-A^{Cse4} mutant leads to its mislocalization (Collins et al., 2004). An E3 ubiquitin ligase Psh1 recognizes the CATD and targets CENP-A^{Cse4} for degradation (Hewawasam et al., 2010; Ranjitkar et al., 2010). Since Scm3 and Psh1 bind to the same domain of CENP-A^{Cse4}, Scm3 protects CENP-A from ubiquitinylation and degradation. The deletion of *SCM3* is lethal, but can be rescued by the overexpression of CENP-A^{Cse4} or deletion of *PSH1.* The CENP-A^{Cse4} turnover is observed even in a *psh1* deletion strain, which implies the existence of additional mechanisms regulating the CENP-A^{Cse4} abundance (Hewawasam et al., 2010). CENP-A^{Cse4}, which is incorporated into the centromeric nucleosome, appears to be protected from degradation by an unknown mechanism. In Drosophila, the Ppa subunit of the E3 ubiquitin ligase SCF targets the CATD of CENP-A^{CID} for degradation (Moreno-Moreno et al., 2011; Moreno-Moreno et al., 2006).

1.12 The timing of CENP-A loading at the centromere

In budding yeast, CENP-A^{Cse4} is loaded onto the centromeres in S-phase coincident with the replication of centromeric DNA (Pearson et al., 2004). Surprisingly, Scm3 peaks at the centromeres in anaphase, i.e. after CENP-A^{Cse4} is loaded, and dissociates from the centromeres after mitosis (Luconi et al., 2011). However, a different group reported that Scm3 localizes to the centromere throughout the cell cycle (Xiao et al., 2011) and the role of Scm3 in loading or protecting centromeric CENP-A^{Cse4} from degradation remains a subject of debate.

In fission yeast, CENP-A^{Cnp1} is loaded on the centromeric DNA in S-phase and in G2. The G2 pathway is replication independent and depends on the Scm3 and Mis16/Mis18 complex (Pidoux et al., 2009; Takahashi et al., 2005). Scm3 transiently dissociates from the centromere from metaphase to early and mid anaphase and is recruited again in late anaphase (Pidoux et al., 2009; Williams et al., 2009). It is worth noting that the fission yeast G1 phase is very short, with the cells rapidly advancing from mitosis into S phase. CENP-A^{Cnp1} loading closely

follows mitosis and correlates with Scm3 localization at the centromere.

In contrast to budding yeast, human CENP-A is very stable. It is produced in late G2 phase (Shelby et al., 2000; Shelby et al., 1997) and is deposited onto chromatin after exit from mitosis in telophase and early G1 (Jansen et al., 2007) at the same time as HJURP (Dunleavy et al., 2009; Foltz et al., 2009). The expression of CENP-A is regulated by a motif in the promoter that is similar to the one found in the promoters of many cell cycle regulated genes (Shelby et al., 1997). Unlike CENP-A, H3 expression peaks in S phase and conventional H3 nucleosomes are assembled during DNA replication (Shelby et al., 1997). Interestingly, CENP-A expressed in S phase from the H3 promoter fails to accumulate at the centromere. This observation is in agreement with the recent discovery that the CENP-A nucleosome assembly factor Mis18BP1 is phosphorylated in a cell cycle dependent manner. The phosphorylation regulates the Mis18BP1 localization to the centromeres. Interestingly, CENP-A is recruited to the centromere throughout the cell cycle when the cyclin dependent kinases Cdk1 and Cdk2 are inhibited (Silva et al., 2012).

In Drosophila syncytial embryonic nuclear divisions, no G1 or G2 phases can be identified and the nuclei cycle between S phase and mitosis. CENP-A^{CID} is deposited at the centromeres not in S phase but in anaphase. Therefore in humans as well as in Drosophila, CENP-A is not loaded at the centromeres during DNA replication and the amount of CENP-A is diluted twofold during S phase (Jansen et al., 2007). It is proposed that nucleosome-free 'gaps' are generated in S phase, which are filled in late mitosis and early G1 or that during the replication H3 nucleosomes are assembled at the centromere and later exchanged for CENP-A nucleosomes. Alternatively, at the end of mitosis CENP-A is deposited onto centromeric chromatin without assembling into nucleosomes and at the time of the DNA replication CENP-A nucleosomes are assembled from this pool (Schuh et al., 2007). It is also possible that heterotypic nucleosomes with one copy each of histone H3 and CENP-A are generated during DNA replication and exchanged for homotypic CENP-A/CENP-A nucleosomes in mitosis (Black and Cleveland, 2011). Yet another hypothesis proposes that the centromeric nucleosomes might split into half-nucleosomes in mitosis, which are then re-constructed into octameric nucleosomes when CENP-A is loaded

(Allshire and Karpen, 2008). Although it is not possible at this moment to distinguish between the proposed scenarios, it is remarkable that assembly of the centromeric nucleosomes is linked to splitting of the sister kinetochores during chromosomal segregation, i.e. to the ultimate "measure" of kinetochore function and therefore ensures the propagation of functional kinetochores through the cell cycles.

1.13 Models of the composition of the CENP-A containing 'nucleosome'

CENP-A is the epigenetic hallmark of centromeres and it was proposed that centromeric nucleosomes have a very special structure. Four different models were proposed. The classical model is the homotypic octamer, where a CENP-A dimer replaces the histone H3 dimer. A homotypic octamer would be very similar to the conventional nucleosome core particle with two copies each of CENP-A, H4, H2A, and H2B (Figure 1.8). The recently solved crystal structures of the CENP-A nucleosome (Tachiwana et al., 2011) and the $(CENP\text{-}A/H4)_2$ heterotetramer (Sekulic et al., 2010) show that CENP-A homodimerizes via a four-helix bundle, and that the H3-H3 and CENP-A-CENP-A interfaces are very similar to each other in the nucleosome structures. In human histone H3, the residues L110, H114, D124, L127, and I131 in the alpha2 and alpha3 helices are responsible for the dimerization via hydrophobic interactions and intermolecular salt bridges between two H3 molecules (Luger et al., 1997). Those residues are conserved between histone H3 and CENP-A and among species. In human CENP-A, those residues correspond to L111, H115, D125, L128, and I132 (Tachiwana et al., 2011) (Figures 1.4, 1.5) formed by the alpha2 and alpha3 helices of its histone fold domain (HFD).

There are some notable differences between the H3 and the CENP-A nucleosome. The CENP-A nucleosome organizes only 121 bp of DNA compared to 146 bp for the H3 nucleosome (Luger et al., 1997; Tachiwana et al., 2011). In the conventional nucleosome the alpha N helix of histone H3 interacts with the ends of the DNA at the entry and exit sites (Luger et al., 1997). The alpha N helix is shorter in CENP-A than in histone H3 (Sekulic et al., 2010; Tachiwana et al., 2011), which explains why less DNA is organized in the CENP-A nucleosome.

The human CENP-A contains a larger loop1 region compared to histone H3. In the CENP-A nucleosome and the $(CENP\text{-}A/H4)_2$ tetramer the loop1 region is exposed and forms a bulge, which comprises arginine 80 and glycine 81 and was proposed to be a binding surface for kinetochore components (Sekulic et al., 2010; Tachiwana et al., 2011). The bulge in loop1 is positively charged in CENP-A, whereas in histone H3 the corresponding surface is negatively charged. The loop1 region is part of the CATD, although deletion of the bulge does not abolish the targeting of CENP-A to the centromere in vivo (Tachiwana et al., 2011). However, the bulge in loop1 is only conserved in mammals and birds (Figure 1.5). The recently solved structure of the budding yeast $(CENP\text{-}A^{Cse4}/H4)_2$ heterotetramer, reveals no positively charged bulge in loop1 and the exposed surface is negatively charged as in the $(H3/H4)_2$ heterotetramer (Cho and Harrison, 2011).

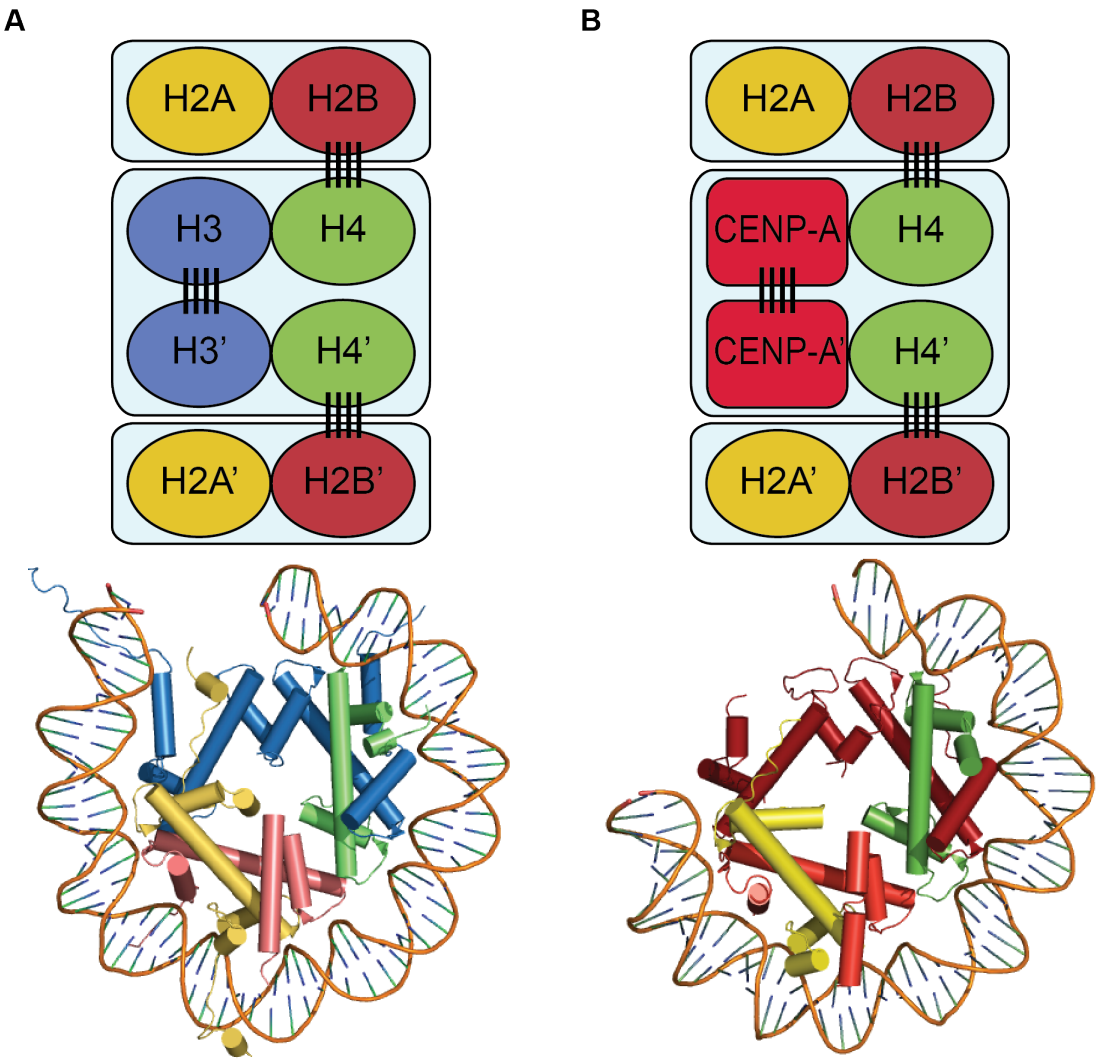

Figure 1.8 The canonical H3 nucleosome and a model of the homotypic CENP-A nucleosome
A) Top: Scheme of the H3 nucleosome with the four-helix bundles between H3 and H3', H2B and H4, and H2B' and H4' indicated by black bars. Bottom: Pymol model of the human H3 nucleosome structure as published in (Luger et al., 1997), shown are the histones H3', H3, H4, H2B and H2A with 73 bp of DNA wrapped. B) Top: Model of the homotypic CENP-A nucleosome. Four-helix bundles are indicated as in (A). Bottom: Pymol model of the human CENP-A nucleosome as published in (Tachiwana et al., 2011), shown are the histones CENP-A', CENP-A, H4, H2B and H2A with 61 bp of DNA wrapped.

There is some evidence that the homotypic CENP-A octamers exist in vivo. In budding yeast expressing two differently tagged versions of CENP-A^{Cse4} both variants could be co-immunoprecipitated (Camahort et al., 2009). A mutation of the residues important for the dimerization of CENP-A^{Cse4} (D217 or L220 to A) abolished the formation of CENP-A^{Cse4} dimers in vivo. In *Drosophila* it was possible to cross-link two CENP-A^{CID} molecules via cysteine residues (C184 and/or C219) in the four-helix bundle. A D211 to A mutation in the alpha3 helix of CENP-A^{CID} reduced the cross-linking efficiency and impaired the centromeric localization of CENP-A^{CID} (Zhang et al., 2012).

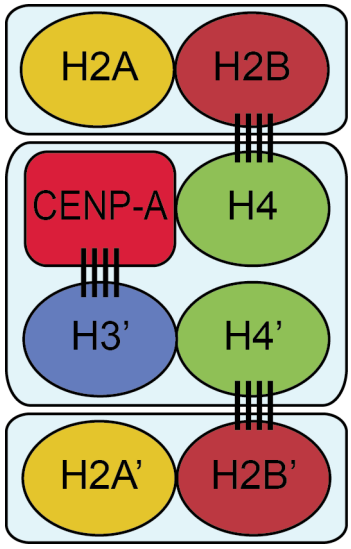

Figure 1.9 The heterotypic CENP-A nucleosome model
Scheme of the heterotypic CENP-A nucleosome with the four-helix bundles between CENP-A and H3', H2B and H4, and H2B' and H4' indicated by black bars.

The heterotypic octamer model proposes that only one, rather than both molecules of H3, is replaced by CENP-A. In the heterotypic octamer, histone H3 and CENP-A are expected to form a four-helix bundle via the alpha2 and alpha3 helices of their HFDs. These residues, which are important for the homodimerization of CENP-A or histone H3, would also be important for the

CENP-A/H3 heterodimerization. There is evidence for the formation of heterotypic octamers on human centromeric DNA in vivo. Purified centromeric mononucleosomes were treated with high salt to dissociate histones H2A and H2B and DNA. Under these conditions CENP-A could be co-immunoprecipitated with H3 and H4 revealing the presence of H3/CENP-A /$(H4)_2$ tetramers along with (CENP-A/H4)$_2$ tetramers (Foltz et al., 2006).

More exotic models of the centromeric nucleosomes were proposed by several laboratories to account for the experimental data that cannot be easily accommodated by the octameric models. When CENP-A^{CID} nucleosomes from Drosophila were cross-linked and separated on a denaturing SDS-PAGE, a heterotetramer comprised of CENP-A^{CID}, H4, H2A, and H2B and not a (CENP-A^{CID}/H4/H2A/H2B)$_2$ octamer was detected (Dalal et al., 2007b). The profile of interphasic CENP-A^{CID} nucleosomes from Drosophila and CENP-A nucleosomes from human, when observed by atomic force microscopy (AFM), appeared half as high as that of canonical nucleosomes (Dalal et al., 2007a; Dalal et al., 2007b; Dimitriadis et al., 2010). CENP-A^{CID} nucleosomes assembled in vitro on a plasmid and CENP-A^{Cse4} nucleosomes assembled on minichromosomes in budding yeast introduce positive supercoils into circular DNA in contrast to canonical nucleosomes, which induce negative supercoils (Furuyama and Henikoff, 2009). The negative supercoiling of circular DNA by canonical nucleosomes is a result of the left-handed wrapping of DNA around the histone octamer. The observed positive supercoiling of the DNA with centromeric nucleosomes was interpreted as an evidence for the right-handed wrapping of DNA in the centromeric nucleosome (Furuyama and Henikoff, 2009). In budding yeast it was shown that the protein footprint at the centromere after a prolonged micrococcal nuclease digest is limited to ~80 bp spanning CDEII (Krassovsky et al., 2012). The so-called hemisome model (Figure 1.10A), proposes a heterotetramer comprised of CENP-A, H4, H2A, and H2B rather than an octamer. It explains why the centromeric nucleosome is half the size of a canonical nucleosome on atomic force microscopy images, as well as the small size of the footprint (~80 bp), and the right-handedness. However, it is important to note that (H3/H4)$_2$ heterotetramers and possibly (CENP-A/H4)$_2$ heterotetramers can wrap DNA in either direction (Hamiche et al., 1996) and

that the left-handed wrap of DNA in an octamer is defined by the addition of H2A/H2B heterodimers (Alilat et al., 1999). It was also observed that the association of H2A/H2B dimers with the $(CENP\text{-}A/H4)_2$ is not as tight as in a canonical nucleosome (Dechassa et al., 2011). The dissociation of H2A and H2B from the centromeric nucleosome in the course of the experiment could potentially lead to positive supercoils of circular DNA. The small footprint over CDEII can be explained not only by a hemisome protecting this area but also by a $(CENP\text{-}A/H4)_2$ heterotetramer, which would organize a comparable length of DNA.

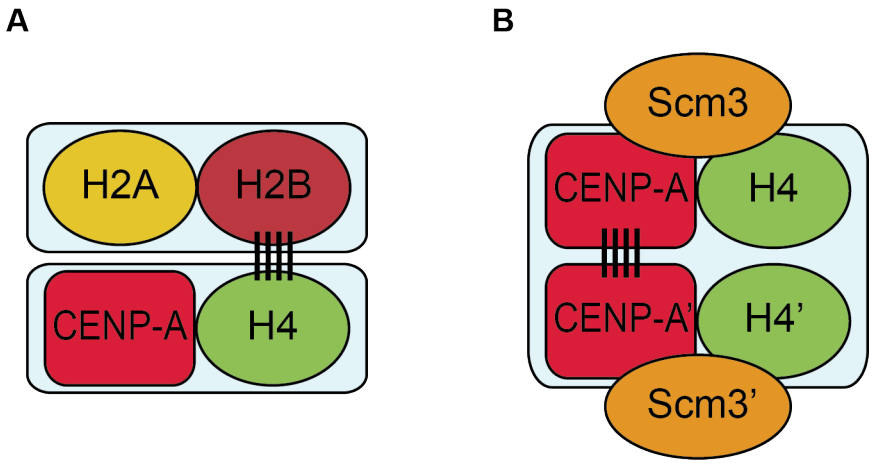

Figure 1.10 The CENP-A hemisome and the CENP-A hexamer model.
A) The hemisome is a tetramer and only consists of one copy each of the histones CENP-A, H4, H2B, and H2A. The hemisome would only organize 61 bp of centromeric DNA. The four-helix bundle between histones H4 and H2B is indicated by four black bars. B) The hexamer is comprised of the CENP-A/H4 homotetramer, and two Scm3 molecules are replacing the H2B/H2A dimers. A highly unlikely four-helix bundle between CENP-A and CENP-A' is indicated by the black bars. See text for further information.

The so-called hexamer model (Figure 1.10B) was proposed to account for the observation that while $CENP\text{-}A^{Cse4}$ and Scm3 map to the centromeric DNA in chromatin immunoprecipitation experiments, the histones H3, H2A and H2B are detected at the centromere at a lower level than on the chromosome arms. Scm3 was reported to replace H2A and H2B from the pre-assembled $CENP\text{-}A^{Cse4}$ octameric nucleosomes in vitro and a stoichiometric complex comprised of $(CENP\text{-}A^{Cse4}/H4/Scm3)_2$ could be reconstituted (Mizuguchi et al., 2007). The $(CENP\text{-}A^{Cse4}/H4/Scm3)_2$ hexamer shows a preference for the AT-rich CDEII region of the budding yeast centromere in assembly reactions in vitro

(Xiao et al., 2011). According to the hexamer model, Scm3 is an integral component of the centromeric nucleosome, which is comprised of (CENP-A^{Cse4}/H4/Scm3)$_2$. However, this model is highly controversial since the discovery that a *scm3* deletion can be rescued by the overexpression of CENP-A^{Cse4} (Camahort et al., 2009). Moreover, it was shown that Scm3 occludes the CENP-A homodimerization interface and prevents the assembly of a (CENP-A/H4)$_2$ heterotetramer. In addition, binding of Scm3 to the CENP-A/H4 dimer impedes the binding of DNA (Bassett et al., 2012; Cho and Harrison, 2011; Hu et al., 2011; Zhou et al., 2011). Therefore it is more likely that Scm3 rather than being an integral component of the centromeric nucleosome plays a role in its assembly and stabilization.

There is evidence for and against each of the abovementioned models. It is possible that regional centromeres with their long arrays of centromeric nucleosomes can accommodate different versions of the CENP-A containing nucleosome. However, all of the suggested models, except for the heterotypic octamer, were also proposed for budding yeast, which have point centromeres.

1.14 The budding yeast centromere assembles a single centromeric nucleosome

The centromeric DNA sequence of budding yeast is ~120 bp long, which is sufficient to accommodate only a single centromeric nucleosome (Meluh et al., 1998). Combining mutations in the histone-fold domain of CENP-A^{Cse4} with base substitutions in CDEI or CDEII regions of the centromere leads to synergistic increases in chromosome loss, whereas combining mutations in CENP-A^{Cse4} and CDEIII does not further increase the missegregation of chromosomes. Based on this genetic evidence, it was proposed that in the single centromeric nucleosome CENP-A^{Cse4} directly interacts with CDEI and CDEII but not with CDEIII, which is occupied by the CBF3 complex (Keith and Fitzgerald-Hayes, 2000).

The hypothesis supporting a single centromeric nucleosome was challenged by chromatin immunoprecipitation/array hybridization (ChIP-on-chip) experiments with CENP-A^{Cse4} and Ndc10, which were reported to spread over an extended peri-centromeric region of 20 kb with a peak over the

centromeric DNA sequence (Riedel et al., 2006). There are certain drawbacks in the classical ChIP approach that might explain the "spreading" of the protein peaks. Proteins might be cross-linked to distant DNA sequences due to the folding of chromatin. The DNA is fragmented by sonication resulting in a fragment size of ~500-1000 bp, which is difficult to control with precision and is much larger than the nucleosomal DNA. Microarray hybridization or PCR analysis cannot provide the information on the length of the original immunoprecipitated DNA fragments. A recent study by (Furuyama and Biggins, 2007) used micrococcal nuclease, which preferentially digests inter-nucleosomal linker, to fragment DNA prior to ChIP and Southern blotting with a short probe specific to the centromeric DNA. A limited micrococcal nuclease digest of centromeric DNA typically yields fragments of 200 bp and the detection by Southern blotting preserves the information about the fragment size. With this approach CENP-A was detected exclusively at the ~200 bp centromeric DNA fragment and not at any flanking regions. However, this view was challenged again in two very recent studies, which quantified CENP-A^{Cse4}-GFP fluorescence in budding yeast. It was reported that 3.5 to 6.0 (Lawrimore et al., 2011) and even up to 7.6 (Coffman et al., 2011) CENP-A^{Cse4}-GFP molecules can be detected on average per centromere in apparent contradiction with the results of (Furuyama and Biggins, 2007). It is important to note that the overexpression and potentially also GFP tagging of CENP-A^{Cse4} could lead to its mislocalization at ectopic sites (Krassovsky et al., 2012), including chromosomal regions immediately adjacent to the centromere. However, it is also possible that the structure of the centromeric 'nucleosome' is very different from the canonical H3 nucleosome and more than two molecules of CENP-A are part of this structure.

1.15 Aim of this study

With the discovery of CENP-A 25 years ago and the observation that this protein is a histone variant, it was postulated that histone H3 is replaced by CENP-A in a special centromeric nucleosome. The interpretation of more recent experimental data resulted in several different models for the composition of

the centromeric nucleosome. In budding yeast, the centromeric nucleosome was studied in vivo primarily using low resolution chromatin immunoprecipitation (ChIP) methods, which were unable to distinguish between the models. Additionally, it remains unclear, which of the CENP-A containing nucleosome-like complexes assembled in vitro corresponds to the centromeric nucleosome in vivo.

We decided to determine the exact composition and localization of the centromeric nucleosome in budding yeast using ChIP protocols that were specifically customized for this purpose. We excised the centromeric DNA fragments with restriction enzymes. ChIP experiments were performed with the core histones H3, H4, H2A, H2B, and CENP-A that were tagged with the same tag to facilitate the direct comparison of the immunoprecipitation efficiencies. The immunoprecipitated DNA was analyzed by Southern blot to detect the excised fragments. If quantitative PCR was employed, the DNA was first size-fractionated to avoid detection of larger fragments. ChIP experiments were performed with and without cross-linker. Using this "multi-method" approach we could show for the first time that contrary to wide spread belief, a canonical histone H3 is not excluded from the centromeric DNA in budding yeast and that the centromeric nucleosome contains both histone H3 and CENP-A.

2 Results

2.1 Contributions

The technical assistant Janina Metzler created the strains 1266 and 1268. The summer student Sona Pirkuliyeva created the strains 1576, 1577, 1587, and 1593 under my supervision. The technical assistant Susanne Hanel created the strains 2561 and 2562 under my supervision.

Dr. Dmitri Ivanov was directly supervising my study and performed the experiment shown in Figure 2.21A. I performed all other experiments. The experiments were designed and analyzed together with Dr. Dmitri Ivanov. A great part of the results presented here was published in (Lochmann and Ivanov, 2012). See appendix for further information.

2.2 A new chromatin immunoprecipitation technique with an improved resolution

To analyze the composition of the centromeric nucleosome in budding yeast earlier studies employed chromatin immunoprecipitation (ChIP) experiments (Camahort et al., 2007; Mizuguchi et al., 2007). In the conventional ChIP approach the proteins are chemically cross-linked to DNA with formaldehyde or glutaraldehyde and the chromatin is sonicated to produce DNA fragment of about 500 bp in size. After the immunoprecipitation the DNA fragments are identified in microarray hybridization assays or by PCR. When applied to the centromere, this conventional ChIP approach suffers certain drawbacks. The fragment size of the DNA is considerably larger than the length accommodated by a canonical nucleosome (146 bp) and the resolution is limited thereby. The resolution problem can be overcome by replacing the sonication step with the micrococcal nuclease treatment, which specifically digests the internucleosomal linker DNA. However, different digest conditions can result in a highly variable size for the kinetochore footprint ranging from 80 bp to 220 bp (Bloom and Carbon, 1982; Funk et al., 1989; Krassovsky et al., 2012). Another problem is the accessibility of the centromeric chromatin by the antibodies, since the efficiency of the co-immunoprecipitation of the

centromeric DNA with the canonical histones is relatively low compared to pericentric regions (Camahort et al., 2007; Camahort et al., 2009; Mizuguchi et al., 2007). In addition, a PCR analysis of the co-immunoprecipitated DNA with a specific pair of primers or a microarray hybridization assay can detect larger DNA fragments without identifying them by size, which further limits the resolution.

We established a novel version of the ChIP technique to reveal the exact composition of the centromeric nucleosome in the budding yeast *Saccharomyces cerevisiae*. There are three significant differences from the conventional ChIP experiments. First, we performed our experiments with and without the chromatin cross-linking. We reasoned that by omitting the cross-linking step the accessibility of the centromeric nucleosome to the antibodies would be improved and potential artifacts caused by cross-linking of loosely associated proteins would be prevented. However, the cross-linking step can also provide certain advantages, e.g. by preventing nucleosomal sliding along the DNA. Therefore, we performed parallel experiments with cross-linking. Second, we introduced restriction sites flanking the centromeric DNA and excised it with a specific endonuclease. Finally, the analysis of the co-immunoprecipitated DNA was accomplished by the use of methods that identify the isolated fragments by size. Initially the DNA was analyzed by Southern blot with probes specifically hybridizing to the excised centromeric DNA fragment. In ChIP experiments, where quantitative PCR (qPCR) with a specific pair of primers was used as a detection method, the co-immunoprecipitated DNA was fractionated by size prior to the qPCR analysis to avoid the detection of uncut DNA (Figure 2.1A). A similar ChIP approach was employed recently by the Biggins's laboratory (Furuyama and Biggins, 2007). In their study, the chromatin was digested with micrococcal-nuclease prior to the immunoprecipitation with an anti-Cse4 antibody and the co-immunoprecipitated DNA was detected by Southern blot. The reported results showed that CENP-A^{Cse4} localizes exclusively to the centromeric DNA in budding yeast but the authors did not address the composition of the centromeric nucleosome further.

2.3 An artificial minichromosome assembles CENP-A^{Cse4} and histone H3 containing nucleosomes

In the initial experiments we employed a small circular minichromosome containing the CEN region of chromosome IV rather than a full chromosome IV (Figure 2.1B). The minichromosome could be specifically co-immunoprecipitated with an anti-HA antibody in the absence of cross-linking from the lysates of the strains with HA-tagged versions of either histone H3 or CENP-A^{Cse4} (Figure 2.2A). This result confirms that the minichromosome assembles canonical nucleosomes as well as a centromeric nucleosome. It also demonstrates that the nucleosomes are associated with the DNA in a sufficiently stable manner to remain bound to the minichromosome in the absence of cross-linking throughout the immunoprecipitation procedure.

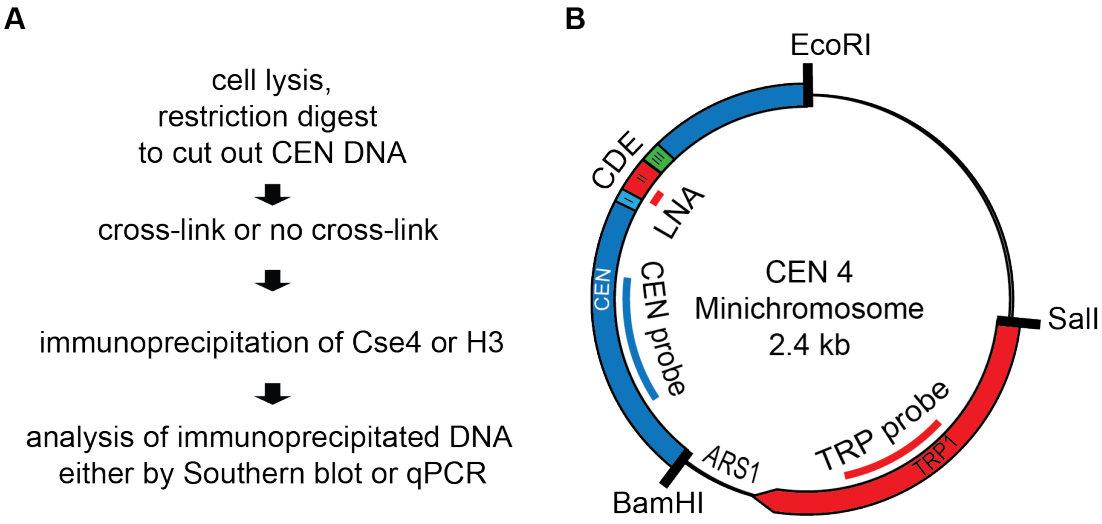

Figure 2.1 A novel ChIP approach with a minichromosome

A) A Scheme of the multi method ChIP approach. B) A Map of the minichromosome. The construct contains 850 bp of pericentromeric DNA from chromosome IV, a *TRP1* marker and the *ARS1* sequence.

It was proposed earlier that CENP-A^{Cse4} localizes not only to a centromere-containing minichromosomes but also to the 2-micron DNA (Hajra et al., 2006; Huang et al., 2011a; Huang et al., 2011b). The 2-micron DNA is a naturally occurring multi-copy extra-chromosomal plasmid with a size of ~6.3 kb (Anders et al., 2006). The plasmid has a copy number of 40-60 per cell and under normal conditions is neither advantageous nor disadvantageous for the host cell (Bilu and Barkai, 2005). The 2-micron plasmid is comprised of four

protein-coding loci (*FLP1*, *REP1*, *REP2* and *RAF1*) and four cis-acting loci (an origin of replication, a partitioning locus, called STB, and two Flp recombinase recognition sequences, called FRTs) (Ahn et al., 1997). The plasmid-coded proteins Rep1 and Rep2 and the STB partitioning locus are required for the stable inheritance of the 2-micron plasmid. CENP-A^{Cse4} was reported to associate with the STB partitioning locus (Huang et al., 2011b). Under our conditions we did not detect a stable association of the 2-micron plasmid with CENP-A^{Cse4} in the immunoprecipitation assay with CENP-A^{Cse4}-HA using anti-HA antibodies and no cross-link (Figure 2.2B). Therefore if CENP-A^{Cse4} is indeed present on the 2-micron DNA it is unlikely to be incorporated into a stable nucleosome.

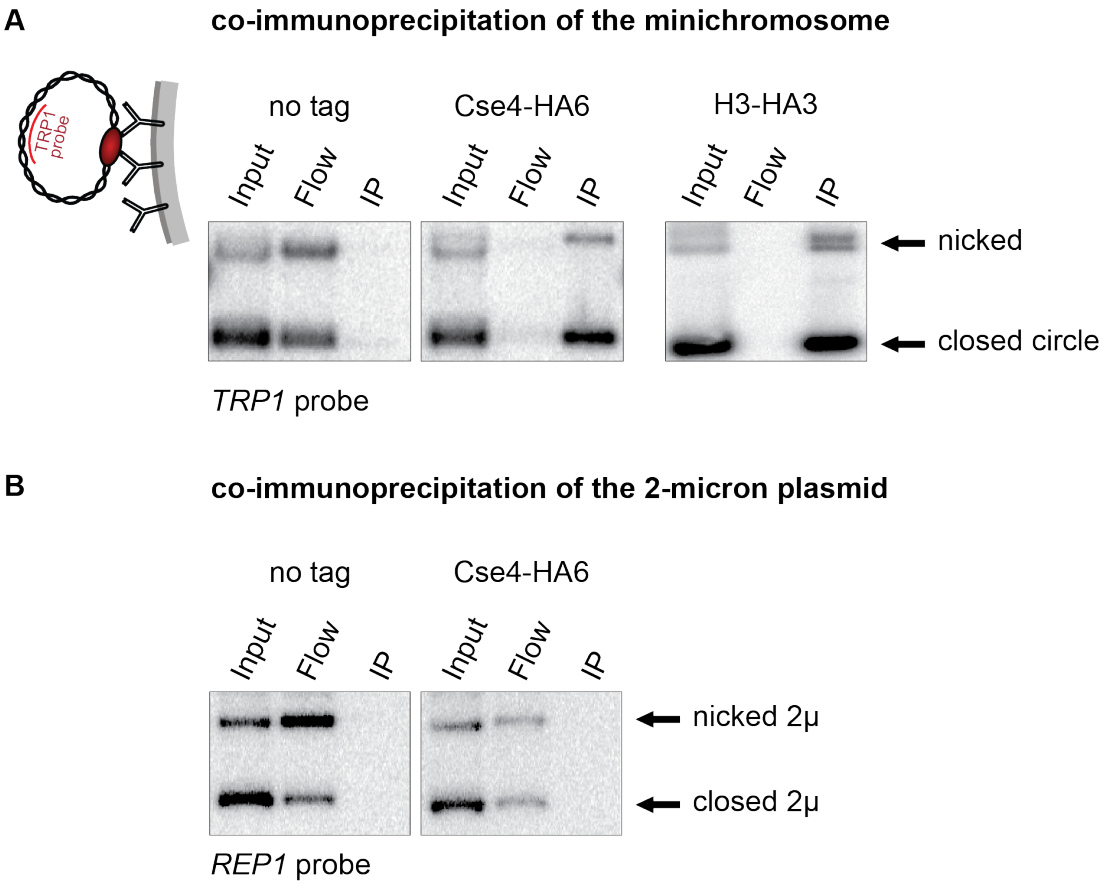

Figure 2.2 The minichromosome assembles Cse4 and H3 nucleosomes

A) The CEN-containing minichromosomes can be specifically co-immunoprecipitated with Cse4 and H3. Lysates from strains transformed with the minichromosomes 1021 (wt), 1498 (Cse4-HA6) and 1407 (H3-HA3) were incubated with anti-HA antibodies and Dynabeads. DNA was eluted off the beads and separated on a 1 % agarose gel. The Southern blot was analyzed using a ^{32}P labeled *TRP1* probe (Lochmann, 2009; Lochmann and Ivanov, 2012). B) The 2micron plasmid is not associated with

Cse4. Shown is the same blot as in A) for no tag and Cse4-HA6 ChIP. The Southern blot was probed with a ^{32}P labeled *REP1* probe.

2.4 A 214 bp CEN fragment is co-immunoprecipitated with CENP-A^{Cse4} and histone H3

Next, we wanted to know whether it is possible to digest the minichromosome in budding yeast cell lysate and subsequently co-immunoprecipitate the resulting minichromosome fragments with the histones. We constructed minichromosomes with BglII sites at various positions with respect to the centromeric DNA and determined the cutting efficiency. The efficiency of digest was very different depending on the position of the BglII site (Figure 2.3A). It was reported earlier that the centromeric DNA is resilient to a nuclease digest (Bloom and Carbon, 1982; Funk et al., 1989). However, under our conditions it was possible to excise the centromeric DNA and even to cut it between CDEII and CDEIII, in agreement with the previous results by (Saunders et al., 1988; Saunders et al., 1990).

In the following ChIP experiments we employed a minichromosome with BglII restriction sites flanking the CEN4 boundaries 50 bp upstream and downstream. Upon digest of chromatin with the endonuclease BglII a 214 bp CEN fragment was released and the digested chromatin was immunoprecipitated with an anti-HA antibody (Figure 2.3B). A probe specifically hybridizing to the *TRP1* gene, which is located on the minichromosome outside of the CEN region, was used for the Southern blot analysis. The restriction digest of crude chromatin is typically incomplete; therefore a CEN-less fragment and a linearized full-length minichromosome could be detected. Only the full-length linearized minichromosome is co-immunoprecipitated with CENP-A^{Cse4}-HA while both the full-length linearized minichromosome and the CEN-less fragment are detected on the beads when the HA-tagged histones H4, H2A, H2B and H3 are immunoprecipitated (Figure 2.3C). Therefore, although the minichromosomes assemble conventional nucleosomes along their entire length, only the centromeric DNA is associated with CENP-A^{Cse4} in agreement with (Furuyama and Biggins, 2007).

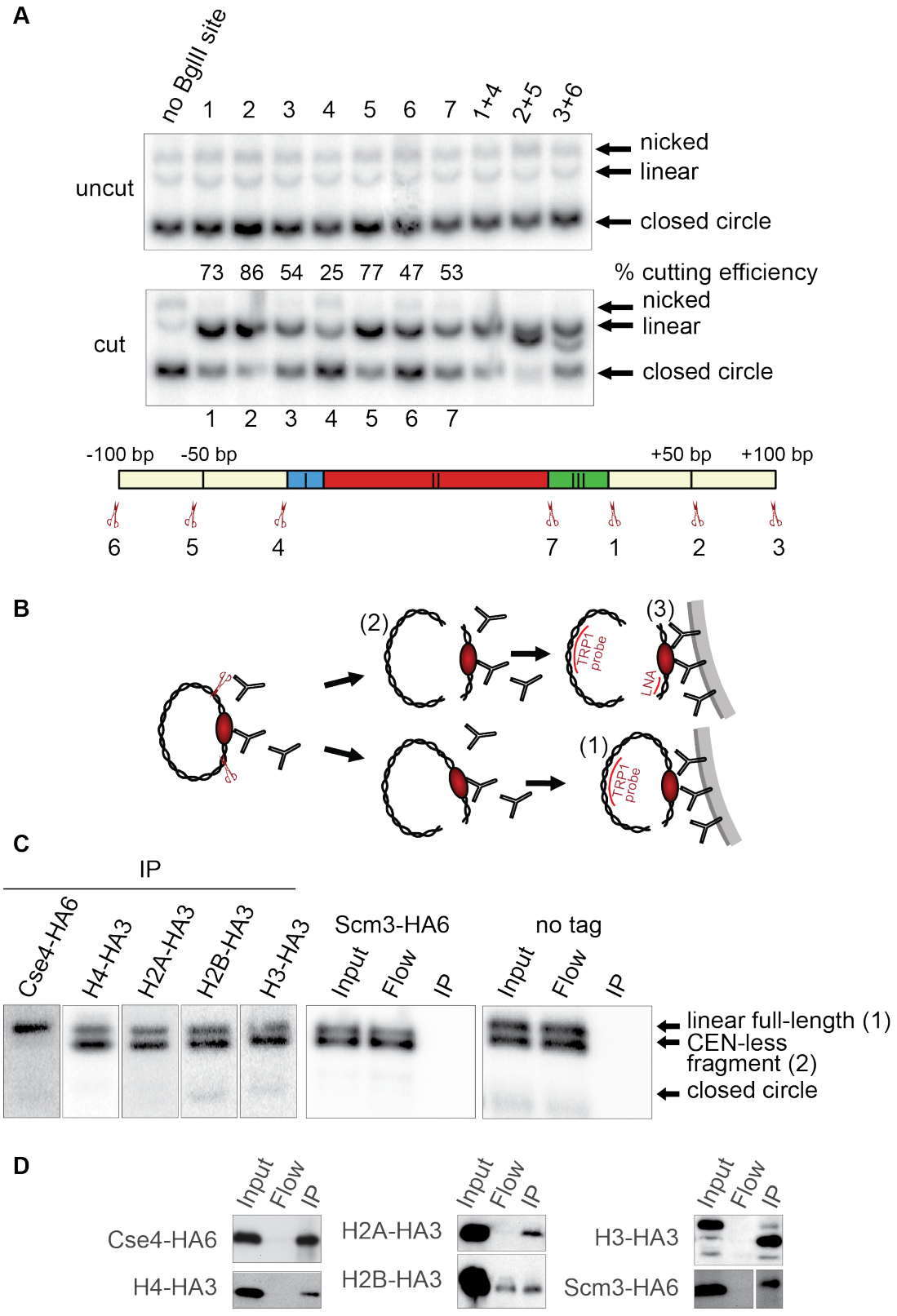

Figure 2.3 Cse4 localizes exclusively to the CEN DNA +/- 50 bp fragment

A) Top: The efficiencies of a minichromosome digest at the indicated sites. The DNA was isolated from BglII-treated lysates of strains carrying minichromosomes with BglII sites at different loci, resolved on a 1 % agarose gel, and analyzed with a ^{32}P labeled *TRP1* probe (Lochmann, 2009). Bottom: Scheme of CEN4 with CDEI, CDEII and CDEIII indicated. The scissors indicate BglII sites in the

different constructs. B) Experimental setup for the immunoprecipitation of minichromosomes digested with BglII. Chromatin is digested with BglII, incubated with anti-HA antibodies recognizing the tagged histones and protein A Dynabeads. A minichromosome digest with BglII produces three different fragments: a linearized full-length minichromosome (1), a CEN-less fragment (2), which can be detected with *TRP1* probe, and a small CEN fragment (3), which can be detected with an LNA oligonucleotide. The red ellipse is depicting the centromeric nucleosome. C) Cse4 binding is restricted to the minichromosomal CEN DNA. BglII-treated chromatin of strains carrying the minichromosome p1009 with BglII restriction sites 50 bp upstream and downstream of the CEN boundaries was immunoprecipitated with anti-HA antibodies. The strains were 1498 (Cse4-HA6), 1577 (H4-HA3), 1576 (H2A-HA3), 1587 (H2B-HA3), 1407 (H3-HA3), 1593 (Scm3-HA6), and 1021 (wt). The DNA was analyzed as in (A) with a ^{32}P labeled *TRP1* probe. D) Anti-HA Western blots of samples from ChIP experiments. Input, unbound fraction, and eluted beads were separated on SDS-PAGE (Lochmann and Ivanov, 2012).

It was proposed recently that the Scm3 histone chaperone might replace H2A/H2B dimers in the centromeric nucleosome (Mizuguchi et al., 2007; Xiao et al., 2011). To test for the presence of Scm3 at the centromeric DNA we performed the minichromosome ChIP experiment with the Scm3-HA6 strain. The minichromosome could not be co-immunoprecipitated with the HA-tagged Scm3 under our conditions. This result indicates that Scm3 is not a stable component of the centromeric nucleosome (Figure 2.3C).

We further attempted to investigate whether it is possible to map the localization of CENP-A^{Cse4} to the exact 111 bp of CEN4, without any flanking sequence. We generated a minichromosome with BglII restriction sites directly at the boundaries of the centromeric DNA and subjecting it to our ChIP approach. With this experimental setup it is not possible to conclude that CENP-A^{Cse4} exclusively localizes to the full-length fragment containing the 111 bp of centromeric DNA. It appears that no CEN-less fragment was co-immunoprecipitated, whereas histone H3 co-immunoprecipitates with both the full length and the CEN-less fragment (Figure 2.4). However, the cutting efficiency for the BglII restriction site at the CDEI boundary was very low, only 25 % as compared to the cutting efficiency of 73 % at the CDEIII boundary (Figure 2.3A) and in the input only the full-length fragment is clearly detectable. Therefore the larger 214 bp centromeric DNA fragment (Figure 2.3C) was employed in further experiments.

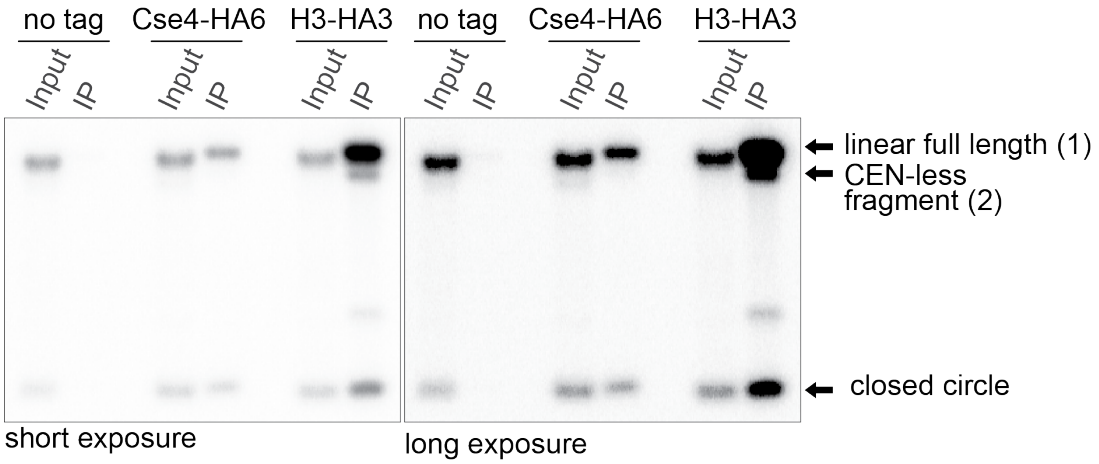

Figure 2.4 Is Cse4 restricted to a 111 bp CEN fragment?
BglII-treated chromatin of strains carrying the minichromosome p1008 with BglII restriction sites directly at the CEN boundaries was immunoprecipitated with anti-HA antibodies. The strains were 1021 (wt), 1498 (Cse4-HA6), and 1407 (H3-HA3). The DNA was resolved on a 1 % agarose gel and the Southern blot was analyzed with a ^{32}P labeled *TRP1* probe. Shown are a short (left) and a long exposure (right) of the Southern blot.

The observation that no CEN-less fragment is immunoprecipitated with CENP-A^{Cse4}-HA6 demonstrates that there is no lateral sliding of the CENP-A^{Cse4} nucleosome during the course of our immunoprecipitation procedure even in the absence of cross-linking (Figure 2.3C). Moreover, the tethering of the DNA fragments via protein-protein interactions, e.g. between centromeric and conventional nucleosomes, could be ruled out in our assay. The minichromosome fragments of approximately 1000 bp and longer could be immunoprecipitated with an exceptionally high efficiency, which was close to 100 %. When a 930 bp fragment from the *ARS1* sequence stopping at a position 50 bp downstream from CDEIII was excised, it was depleted from yeast cell lysate with anti-HA antibodies recognizing CENP-A^{Cse4}-HA6 while practically none of the remaining CEN-less fragment of the minichromosome could be detected on the beads (Figure 2.5). Considering the proximity of the cutting site at +50 bp to the centromere, the absence of the CEN-less fragment in the CENP-A^{Cse4}-HA6 immunoprecipitation in this experiment rules out local sliding of nucleosomes and/or tethering of the fragment with the centromeric DNA sequence to the rest of the minichromosome under our experimental conditions.

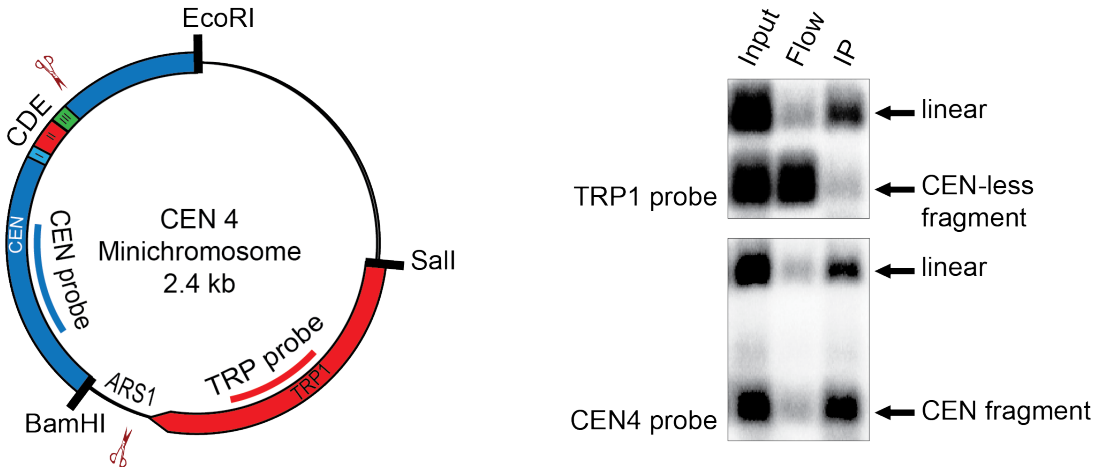

Figure 2.5 The Cse4 nucleosome remains restricted to the CEN DNA in the course of the immunoprecipitation procedure.
Left: A Map of the minichromosome p1171 utilized in the experiment. The construct contains 850 bp of pericentromeric sequence of chromosome IV, a *TRP1* marker and the *ARS1* sequence. BglII restriction sites are located 50 bp downstream of CDEIII and within the *ARS1* and are indicated with scissors. Right: BglII-treated chromatin of a strain 1498 (Cse4-HA6) carrying the minichromosome shown in A) was immunoprecipitated with anti-HA antibodies without cross-linking. A long version of the procedure with 2 hours restriction digest was used. The DNA was separated on a 1 % agarose gel, the Southern blot was analyzed with a *TRP1* probe to detect the CEN-less fragment and a CEN4 probe hybridizing to the pericentromeric sequence to detect the fragment of the minichromosome containing CEN4 (Lochmann, 2009; Lochmann and Ivanov, 2012).

Whereas the linear minichromosome and the CEN-less fragment could be detected easily with ^{32}P-labelled probes, the detection of the small 214 bp CEN fragment (Figure 2.6A,B) was very inefficient using this approach. Thus we used a digoxygenin (DIG)-labeled locked nucleic acid (LNA) oligonucleotide with enhanced hybridization properties (Petersen and Wengel, 2003). Employing this LNA probe we could detect the 214 bp CEN fragment excised from 6 pg of the minichromosome, which corresponds to a detection efficiency of approximately 0.1 % in an immunoprecipitation experiment starting with 150 ml of a budding yeast cell culture in the early log phase (Figure 2.6C). Using this novel detection method for Southern blots, we observed the 214 bp CEN fragment in the immunoprecipitates with CENP-A^{Cse4}, H4, H2A and H2B. Unexpectedly, we reproducibly detected the small CEN fragment in the immunoprecipitates with histone H3 as well (Figure 2.6D). This observation is in contrast to several previous studies, which suggested that histone H3 is replaced by CENP-A^{Cse4} at the centromere (Meluh et al., 1998; Shelby et al., 1997; Sullivan et al., 1994) and contradicts an “established dogma” in the field.

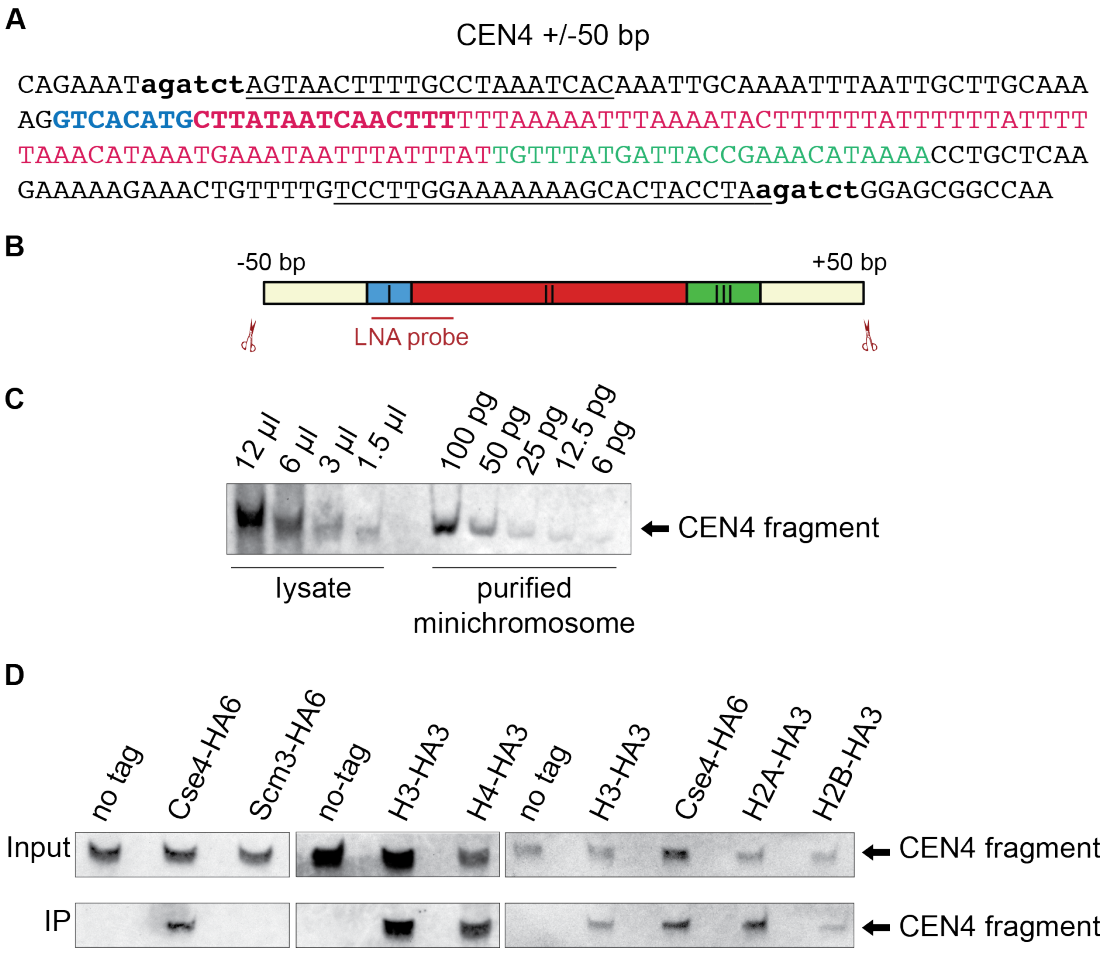

Figure 2.6 The centromeric DNA is associated with Cse4 as well as histone H3
A) The DNA sequence of CEN4 with BglII sites at +/- 50 bp. CDEI, II, and III are shown in blue, red and green respectively. BglII site are indicated in bold lowercase. The double DIG-labeled LNA probe (recognizes CDEI and a part of CDEII) is depicted in bold uppercase. Underlined are the sequences where the qPCR primers anneal. B) A Scheme of the excised CEN fragment. The double-DIG labeled LNA probe for CDEI/II is indicated. C) Sensitivity of the Southern blot detection with the double DIG-labeled LNA probe for CDEI/II. DNA purified from BglII-treated lysate of a strain 1021 (wt) carrying the minichromosome p1009 with BglII restriction sites 50 bp upstream and downstream of CEN4 and known quantities of the minichromosome purified from bacteria (miniprep) were digested with BglII, resolved on a 6 % denaturing TBE polyacrylamide gel, and analyzed by Southern blot with the LNA probe for CDEI/II. D) Histone H3 localizes to the centromeric DNA. The immunoprecipitated DNA from experiments shown in Figure 2.3C was separated on a 6 % denaturing TBE polyacrylamide gel. The Southern blot was analyzed using a double-DIG labeled LNA probe for CDEI/II (Lochmann and Ivanov, 2012).

Mammalian centromeric chromatin and the flanking heterochromatin were found to be associated with the histone H2A variant H2A.Z (Greaves et al., 2007). To investigate whether the H2A.Z homologue in budding yeast (Htz1) is localized to centromeric DNA, we subjected a strain with an HA tagged version of H2A.Z^{Htz1} carrying the minichromosome to our ChIP approach. A minor fraction of minichromosomes is associated with H2AZ^{Htz1} and can be

immunoprecipitated with low efficiency using anti-HA antibodies recognizing the HA-tag of H2A.Z^{Htz1}. Upon restriction digest with BglII and subsequent immunoprecipitation, both the CEN-less and the CEN-containing fragment are detected in the immunoprecipitate (Figure 2.7). However, we could not observe a stable association of H2A.Z^{Htz1} with the 214 bp centromeric DNA fragment in budding yeast (data not shown).

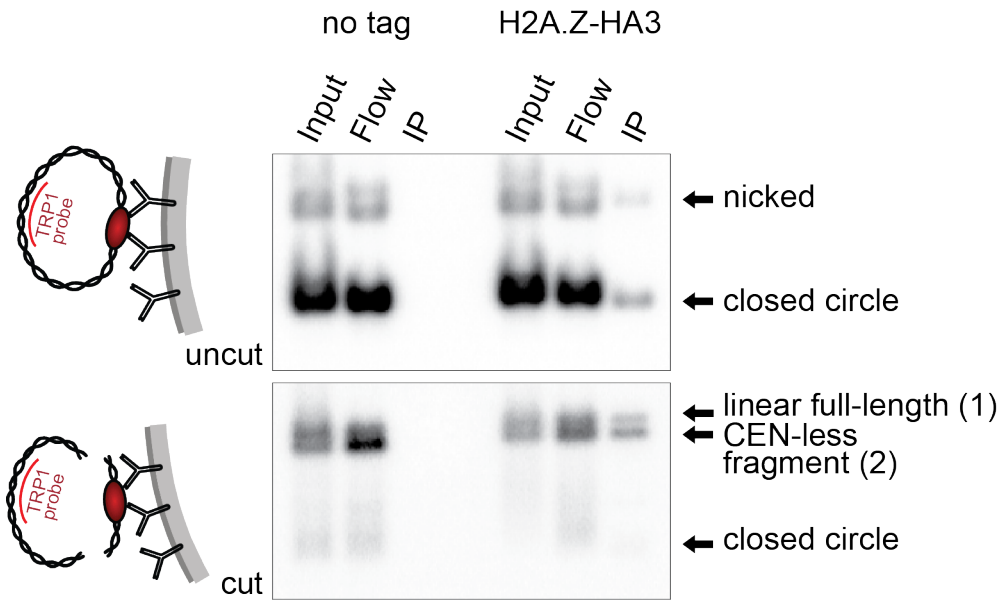

Figure 2.7 The CEN-containing minichromosomes can be specifically co-immunoprecipitated with H2A.Z-HA3

Chromatin from strains transformed with the minichromosomes (p1009) 1021 (wt) and 2070 (H2A.Z-HA3) were either not digested or digested with BglII and incubated with anti-HA antibodies and Dynabeads. DNA was eluted off the beads and separated on a 1 % agarose gel. The Southern blot was analyzed using a ^{32}P labeled *TRP1* probe.

2.5 Histone H3 and CENP-A^{Cse4} localize to the centromeric DNA throughout the cell cycle

It has been proposed earlier that the composition of the centromeric nucleosome might change during the progression of the cell cycle (Black and Cleveland, 2011; Bui et al., 2012; Shivaraju et al., 2012; Xiao et al., 2011). We tested whether the interaction of histone H3 with the CEN fragment is dependent upon the cell cycle as it is possible that CENP-A^{Cse4} replaces histone H3 at a specific stage. The budding yeast cell cultures were arrested in G1 phase with alpha-factor and in G2 phase of the cell cycle with nocodazole/benomyl. To release the 214 bp CEN fragment prior to the immunoprecipitation, the

chromatin was digested with BglII. We observed that histone H3 and CENP-A^{Cse4}, as well as histone H2B, are associated with the CEN fragment in G1 phase as well as in G2 phase of the cell cycle (Figure 2.8). This result is consistent with a continuous occupancy of the centromere by histone H3 rather than with a transient replacement.

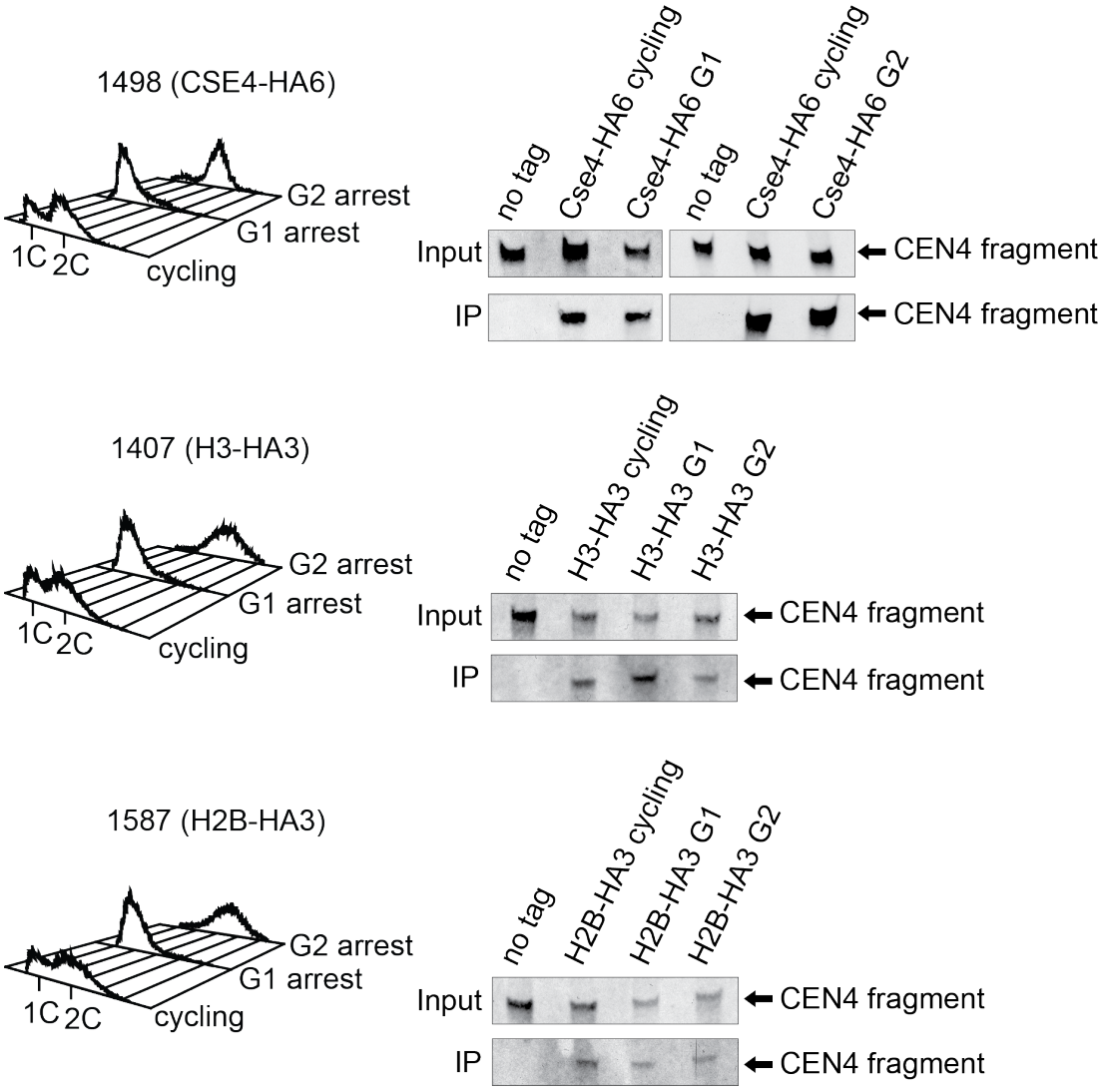

Figure 2.8 Histone H3 is associated with the CEN DNA fragment throughout the cell cycle.
The strains carrying the minichromosomes p1009 with BglII restriction sites 50 bp upstream and downstream of CEN boundaries, 1498 (Cse4-HA6), 1407 (H3-HA3), and 1587 (H2B-HA3) were arrested in G1 with alpha factor and in G2 with nocodazole/benomyl. Chromatin was treated with BglII and immunoprecipitated with anti-HA antibodies. The DNA was eluted off the beads and resolved on a 6 % denaturing TBE polyacrylamide gel. The Southern blot was analyzed with a double-DIG labeled LNA probe for CDEI/II. The FACS analyses of the arrested yeast cultures are shown on the left (Lochmann and Ivanov, 2012).

2.6 Histone H3 and CENP-A^{Cse4} localize to a 214 bp fragment excised from the native chromosome IV

It is possible that a fraction of minichromosomes assembles a canonical H3 nucleosome at the centromere, while the centromeres of other minichromosomes assemble CENP-A^{Cse4} nucleosomes. This might explain the observed association of histone H3 with the centromeric DNA in the above experiments. However, a close to 100 % efficiency of co-immunoprecipitation of the minichromosomes with CENP-A^{Cse4}-HA6 (Figure 2.2A) indicated that it is unlikely to be the case. Nevertheless, to address this possibility we changed our ChIP approach to investigate the native centromeres on the chromosomes and inserted BglII restriction sites 50 bp upstream and downstream of the centromeric DNA on chromosome IV (at the same positions as on the minichromosome, Figure 2.6A). After digesting chromatin with BglII, the released 'native' 214 bp CEN4 fragment was efficiently co-immunoprecipitated with H3-HA3 and CENP-A^{Cse4}-HA6 (Figure 2.9). We conclude that in budding yeast both H3 and CENP-A^{Cse4} localize to centromeric DNA.

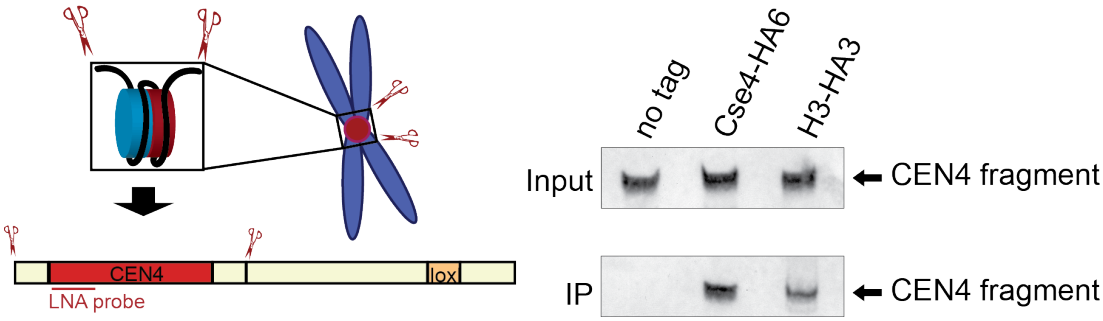

Figure 2.9 Histone H3 is associated with the CEN DNA on the native chromosome IV
BglII-treated chromatin of strains with BglII sites 50 bp upstream and downstream of CEN boundaries on chromosome IV 2059 (wt), 2043 (Cse4-HA3), and 2042 (H3-HA3) was immunoprecipitated with anti-HA antibody. The DNA was eluted off the beads, separated on a 6 % denaturing TBE polyacrylamide gel and analyzed with a double-DIG labeled LNA probe for CDEI/II (Lochmann and Ivanov, 2012).

2.7 Histone H3 is not turned over or sliding laterally during the immunoprecipitation procedure

It is possible that histone H3 replaces CENP-A^{Cse4} in the course of our immunoprecipitation procedure. To rule out this possibility, we combined the yeast cell lysate of a strain with a tagged histone H3 (H3-HA3) that does not

harbor minichromosomes with the lysate of a strain where histone H3 is not tagged (wild type) harboring the minichromosomes. No minichromosomes could be immunoprecipitated from the mixed lysate with the anti-HA antibody (Figure 2.10). We conclude that there is little or no turnover of histone H3 associated with the minichromosomes in our yeast cell lysates.

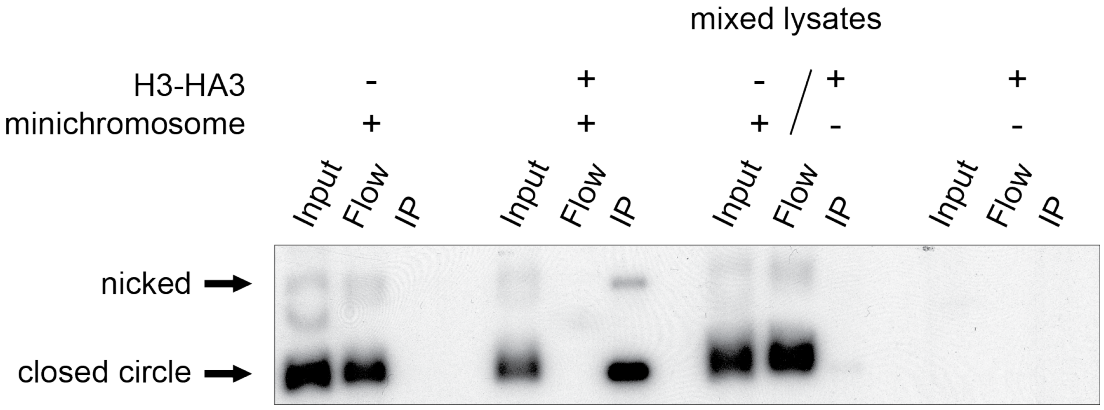

Figure 2.10 Minichromosome-bound histone H3 does not turn over during the immunoprecipitation procedure

Lysates of strains 1021 (wt, carrying the minichromosome), 1407 (H3-HA, carrying the minichromosome), 1407 (H3-HA3, without the minichromosome), and mixed lysate of 1021 (wt with minichromosome) and 1407 (H3-HA3, without the minichromosome) were incubated with anti-HA antibody and Dynabeads. The DNA was eluted off the beads, separated on a 1 % agarose gel, and analyzed using a ^{32}P labeled *TRP1* probe (Lochmann and Ivanov, 2012).

This experiment does not rule out the local rearrangement of nucleosomes, such as lateral sliding, in the course of our experimental procedure. Since our experimental approach included long incubations we decided to cross-link proteins to DNA with formaldehyde before the immunoprecipitation step. The addition of formaldehyde to the spheroplasts severely reduced the co-immunoprecipitation efficiency of the minichromosome with either CENP-A^{Cse4} or histone H3. The low efficiency of immunoprecipitation might be, at least in part, due to the loss of minichromosomes from the lysate during the centrifugation step, for clearing the lysate, probably because of the cross-linking of minichromosomes to larger cellular structures (Figure 2.11A). However, the addition of formaldehyde immediately following the yeast cell lysis did not impede the subsequent immunoprecipitation (Figure 2.11A). To minimize the potential rearrangement of nucleosomes after and during the cell disruption, the time of the digest of the

chromatin with BglII was reduced to 5 minutes at 37°C and subsequently formaldehyde was added. Cutting and the immunoprecipitation of the large fragments of the minichromosome was not impaired compared to the earlier experimental setup with a restriction digest of chromatin for 2 hours a 4°C (Figure 2.11B). Importantly, after introducing the cross-linking step we could still efficiently co-immunoprecipitate the 214 bp CEN fragment of the minichromosome with both CENP-A^{Cse4} and histone H3 (Figure 2.12). Consequently, the detection of histone H3 at the CEN DNA is unlikely to be an experimental artifact of nucleosomal sliding under our conditions.

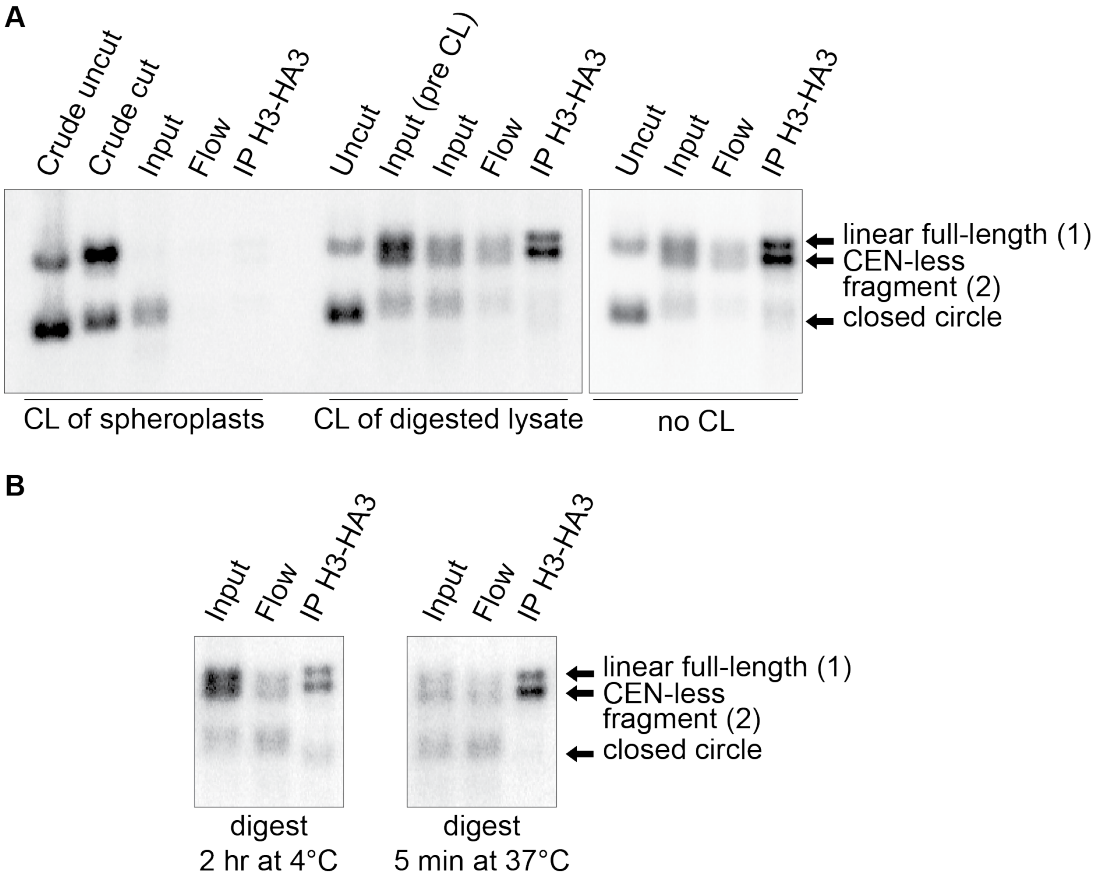

Figure 2.11 Experimental setup of ChIP with formaldehyde cross-linking and short chromatin digest

A) Cross-linking of spheroplasts impairs immunoprecipitation efficiency. A strain 1407 (H3-HA3) carrying the minichromosome p1009 with BglII restriction sites 50 bp upstream and downstream of CEN boundaries was used. Left part: Spheroplasts were cross-linked with 1% formaldehyde, lysed, chromatin was digested, and the lysate was cleared by centrifugation. Middle part: Cells were lysed and the lysate was cleared by centrifugation. The chromatin was digested with BglII and cross-linked with 0.1 % formaldehyde. Right part: Same as in the middle but without cross-linking. The prepared lysates were incubated with anti-HA antibodies and Protein A Dynabeads. The DNA was separated on a 1 % agarose gel and the Southern blot was analyzed using a ^{32}P labeled *TRP1* probe. B) A short

BglII digest is sufficient to fragment the minichromosome. Same strain as in (A). The cells were lysed and the lysate was cleared by centrifugation. The chromatin was digested with BglII either for 2 hours at 4°C (left) or for 5 min at 37°C and cross-linked with 0.1 % formaldehyde. The prepared lysates were incubated with anti-HA antibodies and Protein A Dynabeads. The DNA was separated on a 1 % agarose gel and the Southern blot was analyzed using a ^{32}P labeled *TRP1* probe.

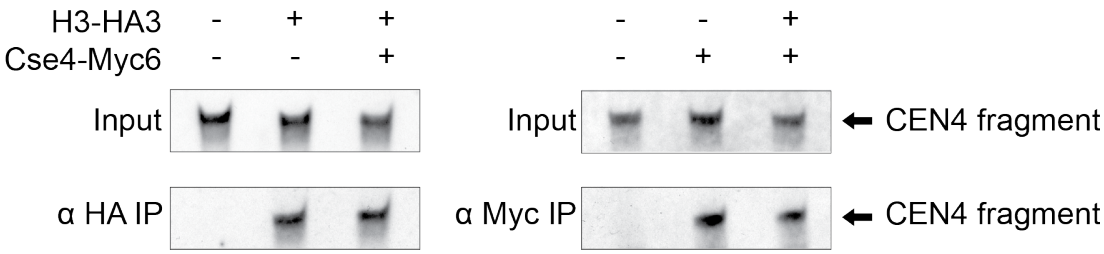

Figure 2.12 ChIP of the 214 bp CEN-fragment with cross-linking
BglII-treated chromatin of the strains 1021 (wt), 1407 (H3-HA3), 1923 (Cse4-Myc6), and 2300 (H3-HA3, Cse4-Myc6) carrying the minichromosome was cross-linked with formaldehyde and immunoprecipitated with anti-HA or anti-Myc antibodies. The DNA was eluted off the beads, resolved on a denaturing 6 % polyacrylamide gel and analyzed with a LNA probe for CDEI/II (Lochmann and Ivanov, 2012).

2.8 The centromeric DNA is co-immunoprecipitated with histone H3 and CENP-A^{Cse4} with similar efficiencies

A qPCR-based approach was used to compare the efficiencies of co-immunoprecipitation of the centromeric DNA with H3-HA3 and CENP-A^{Cse4}-HA6. After releasing the 214 bp CEN fragment by restriction digest, chromatin was immunoprecipitated with anti-HA antibodies recognizing CENP-A^{Cse4}-HA or H3-HA. DNA was eluted off the beads with a buffer containing 1% SDS, size-fractionated via agarose gel-electrophoresis to separate the small fragment (Figure 2.6A) from the full-length minichromosome and quantified by a quantitative PCR (qPCR) reaction with a specific pair of primers (Figure 2.13A). Using this approach, we confirmed that the 214 bp CEN fragment was efficiently separated from the full length minichromosome during the gel-fractionation step since no PCR product was obtained in the control experiment where the restriction digest step was omitted (Figure 2.13B). Interestingly, we could not detect any substantial differences in the efficiencies of CEN DNA ChIP when histone H3 and CENP-A^{Cse4} were immunoprecipitated with the same anti-HA antibody. The IP/input ratios were similar between the H3-HA and CENP-A^{Cse4}-HA strains regardless of whether the experiment was performed with or

without cross-linking, with the centromeric DNA fragment excised from a minichromosome or from the native chromosome IV (Figure 2.13C). This result argues that histone H3 associates with a substantial fraction, if not all, of the centromeres and not just a minor fraction of them.

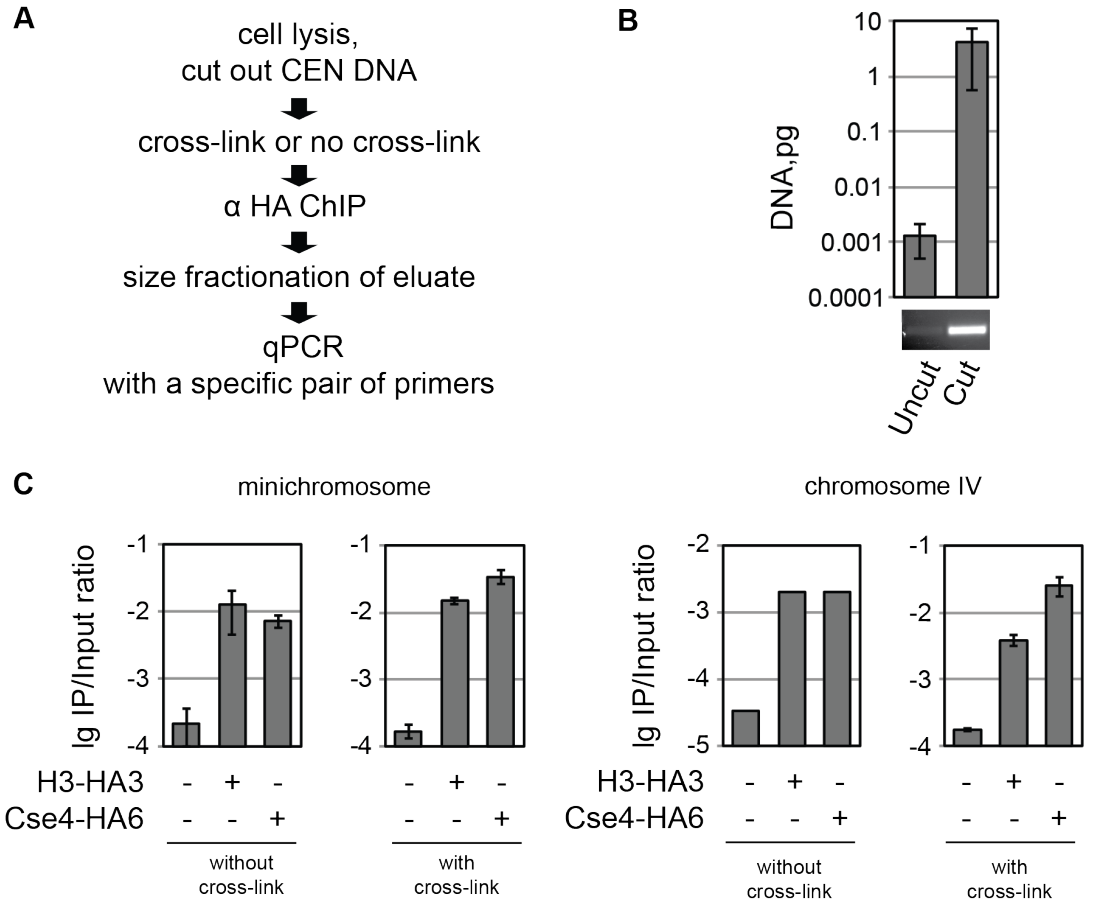

Figure 2.13 ChIP/qPCR of the minichromosomal and the native CEN DNA fragment after formaldehyde cross-link

A) A Scheme of the experimental setup of the ChIP/qPCR approach. B) Only the 214 bp CEN4 fragment and no full-length minichromosome is detected in the ChIP/qPCR assay. The DNA isolated from untreated and BglII-treated lysates was size-fractionated on a 2 % agarose gel and analyzed by qPCR. A PCR product after 30 cycles of amplification in a conventional PCR reaction with the same primers that were used for qPCR is shown below. C) The minichromosomal CEN DNA and CEN DNA of the native chromosome IV can be co-immunoprecipitated with histone H3 and Cse4. BglII-treated chromatin of the strains 1021 (wt), 1407 (H3-HA3), and 1498 (Cse4-HA6) carrying the minichromosome p1009 and the strains 2059 (wt), 2042 (H3-HA3), and 2043 (Cse4-HA6) with CEN DNA of the native chromosome IV flanked with BglII were either not cross-linked or cross-linked with formaldehyde and immunoprecipitated with anti-HA antibodies. The immunoprecipitated DNA was purified, size fractionated, and subjected to qPCR analysis. The bar graphs represent the average values from several independent experiments with SDs (Lochmann and Ivanov, 2012).

2.9 Histone H3 and CENP-A^{Cse4} co-occupy the centromeric DNA

It is possible that the association of histone H3 and CENP-A^{Cse4} with centromeric DNA is mutually exclusive, i.e. some yeast centromeres are occupied by a canonical H3 nucleosome while others assemble CENP-A^{Cse4} nucleosomes. An alternative is that histone H3 and CENP-A^{Cse4} are co-occupying the centromeric DNA simultaneously. To discriminate between these two possibilities we established a sequential ChIP experiment. After the release of the 214 bp CEN fragment by a restriction digest and cross-linking with formaldehyde, the chromatin was immunoprecipitated with anti-Myc antibodies recognizing CENP-A^{Cse4}-Myc. The antibodies in this experiment were covalently coupled to the beads. The immunoprecipitate was eluted off the beads with 1% SDS, diluted to 0.1 % SDS and subsequently re-immunoprecipitated with H3-HA using anti-HA antibodies. The cross-link of DNA recovered from the beads was reverse and the DNA was size-fractionated using agarose gel-electrophoresis. To quantify the eluted centromeric DNA, a qPCR reaction with a specific pair of primers was employed (Figure 2.14A). The second immunoprecipitation step in this experiment exhibited an approximately 100 fold higher efficiency compared to the mock immunoprecipitation from a strain where only CENP-A^{Cse4} was tagged and was similar to the efficiency of a H3-HA re-immunoprecipitation in an experiment where both the first and the second immunoprecipitation were carried out with anti-HA antibodies. Comparable results were obtained when the centromeric DNA was excised from the minichromosome (Figure 2.14B) or the native chromosome IV (Figure 2.14C). Thus, at least for some centromeres, histone H3 and CENP-A^{Cse4} co-exist. Unfortunately, it was not possible to perform the reciprocal experiment, i.e., to immunoprecipitate the centromeric DNA via HA-tagged histone H3 and then re-immunoprecipitate with CENP-A^{Cse4} via the Myc-tag. The anti-Myc antibody did not perform well in 0.1 % SDS. Changing the tags, i.e., CENP-A^{Cse4} -HA and H3-Myc, failed due to the inviability of the H3-Myc6 strain. We also tried to tag histone H3 with a FLAG epitope. The H3-FLAG strain was viable, however the immunoprecipitation efficiency of the full-length minichromosome was too low for a sequential ChIP experiment (Figure 2.15).

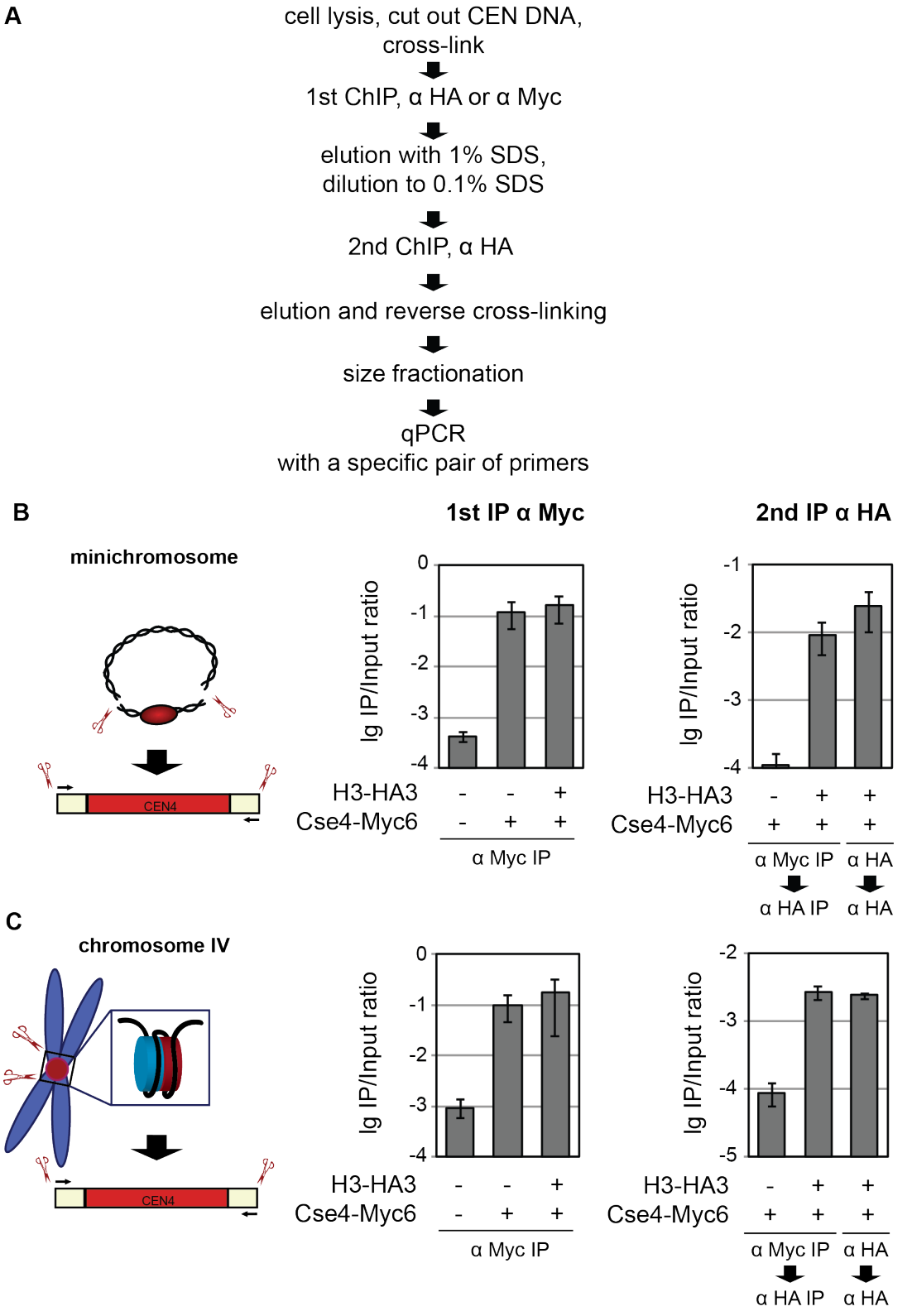

Figure 2.14 Co-occupancy of the centromeric DNA by histone H3 and Cse4

A) A scheme of the sequential Cse4-H3 ChIP. B) Sequential ChIP of minichromosomal CEN DNA. BglII-treated chromatin of the strains 1923 (Cse4-Myc6) and 2300 (H3-HA3, Cse4-Myc6) carrying the minichromosome p1009 was cross-linked with formaldehyde and immunoprecipitated with anti-Myc antibody (1st IP see middle panel) or anti-HA antibody (1st IP see Figure 2.13C) as indicated in the figure. The DNA was eluted off the beads and re-immunoprecipitated with anti-HA antibodies (2nd IP

right panel). The immunoprecipitated DNA was purified, size fractionated on a 2 % agarose gel, and subjected to qPCR analysis. C) The same as in (B) but performed with the native CEN DNA. The strains, 2562 (Cse4-Myc6) and 2561 (H3-HA3, Cse4-Myc6) had CEN DNA of the native chromosome IV flanked with BglII. The bar graphs represent the average values from several independent experiments with SDs (Lochmann and Ivanov, 2012).

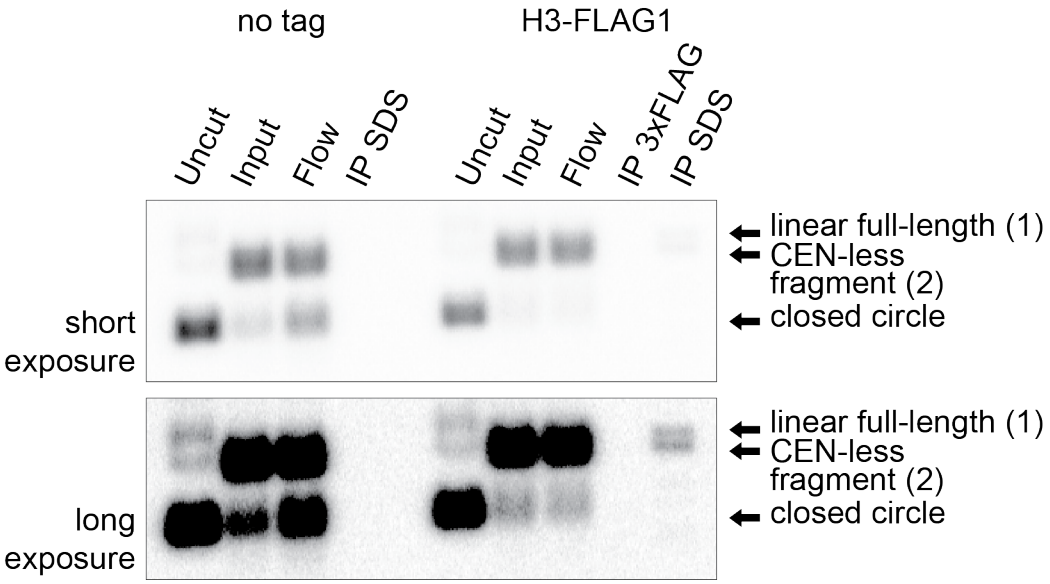

Figure 2.15 Low efficiency of co-immunoprecipitation of the minichromosome with H3-FLAG1
BglII-treated chromatin of strains carrying the minichromosome p1009 with BglII restriction sites 50 bp upstream and downstream of CEN boundaries was immunoprecipitated with anti-FLAG antibody and Protein G Dynabeads. The strains were 1021 (wt) and 2202 (H3-FLAG1). The DNA was either eluted with the 3xFLAG-peptide (IP 3xFLAG) or with SDS (IP SDS) and was analyzed by separating on a 1% agarose gel and Southern blot, which was hybridized with a ^{32}P labeled *TRP1* probe.

2.10 Is the centromeric nucleosome a heterotypic octamer?

Because the length of our excised centromeric DNA fragment is only 214 bp, it is considerably shorter than the 292 bp necessary to accommodate two canonical nucleosomes (assuming no linker DNA in-between). It is also shorter than 268 bp, which would accommodate a combination of a canonical nucleosome and a CENP-A^{Cse4} nucleosome assuming that the latter organizes only 121 bp of DNA (Tachiwana et al., 2011). Therefore it is reasonable to assume that the centromeric nucleosome is a heterotypic octamer with a single molecule each of H3 and CENP-A^{Cse4}. If the structure of this theoretical heterotypic nucleosome is comparable to the structures of the canonical H3 nucleosome and the CENP-A containing nucleosome (Luger et al., 1997; Tachiwana et al., 2011), the histones H3 and CENP-A^{Cse4} are predicted to build a four-helix bundle with parts of their α2 and α3 helices. To test for the formation

of the H3/CENP-A^{Cse4} four-helix bundle, we exploited a cysteine cross-link approach. The α2 helix of H3 has a cysteine residue, C111 in many vertebrates and various other organisms (Figure 2.16B).

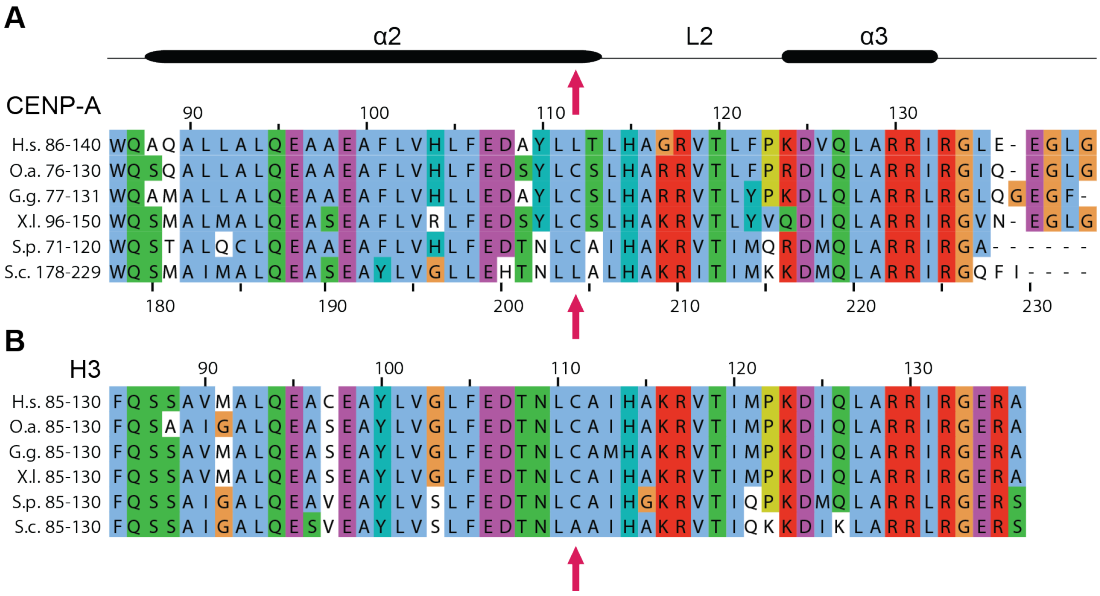

Figure 2.16 The cysteine residue in the alpha2 helix is conserved in CENP-A and histone H3 homologues among species

A) Shown is the ClustalW (www.ebi.ac.uk/Tools/msa/clustalw2/) alignment of the C-terminal ~50 amino acid of CENP-A from different species (*Homo sapiens* (H.s.), *Ornithorhynchus anatinus* (O.a.), *Gallus gallus* (G.g.), *Xenopus laevis* (X.l.), *Schizosaccharomyces pombe* (S.p.) *Saccharomyces cerevisiae* (S.c)) prepared with JalView 2.6.1. and the clustalX color setting. The secondary structure is indicated on top: an alpha helix is indicated by a α and a loop by L. The red arrow indicates the position of the cysteine residue. B) Same as in (A) but with homologues of histone H3.

The distance between the cysteine residues from two histones H3 within the human nucleosome is 6.2 Å (Luger et al., 1997) and under oxidizing conditions avian histone H3 molecules were reported to form a disulfide bond in vitro (Camerini-Otero and Felsenfeld, 1977). The corresponding residue in human CENP-A is a leucine, L112. However, CENP-A homologues from several other mammals, such as *Ornithorhynchus anatinus* (platypus), as well as birds and amphibians contain a cysteine in this position (Figure 2.16A).

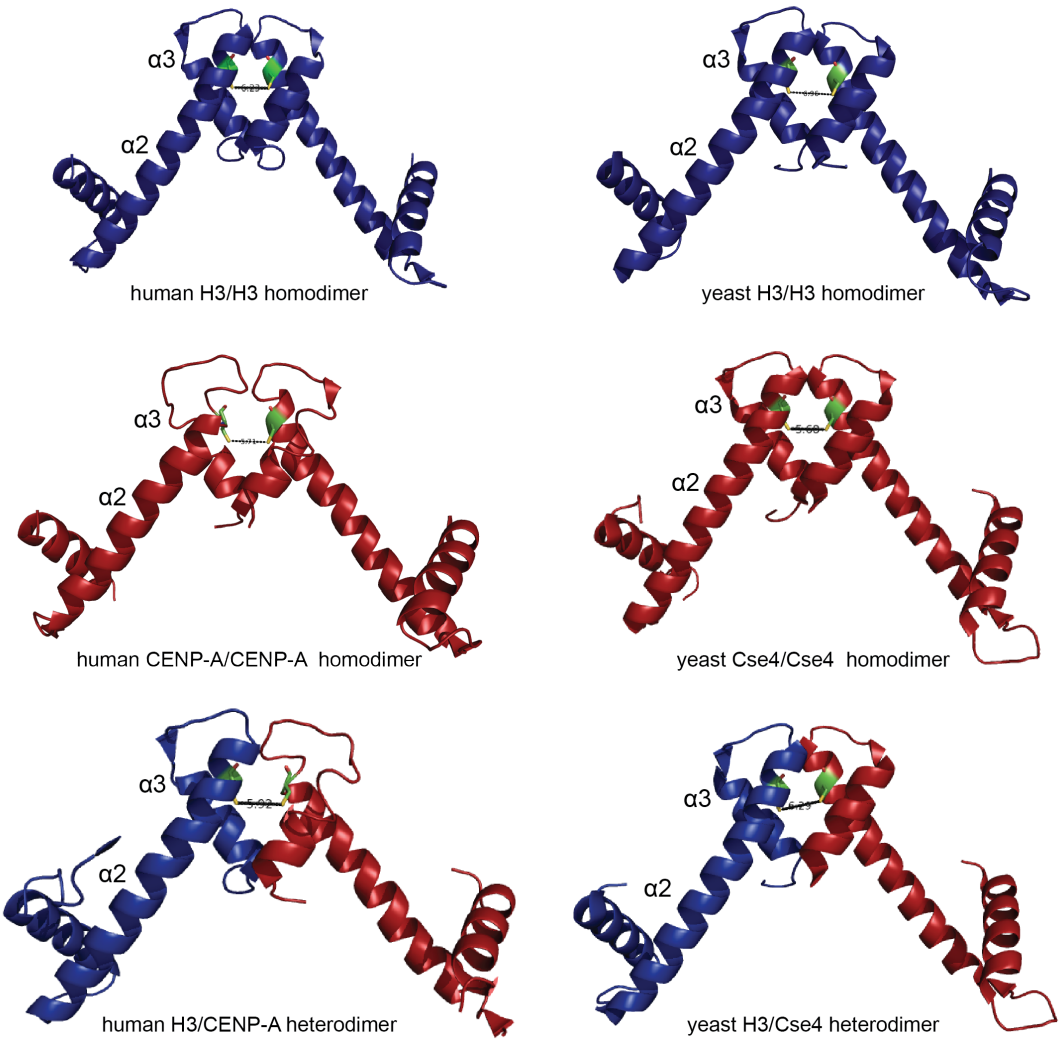

Figure 2.17 Structure of the four-helix bundle of the human and yeast histone H3 homodimer, the CENP-A homodimer and the H3/CENP-A heterodimer
Left panel: Pymol models of the human the histone H3 histone fold domain and the CENP-A histone fold domain with leucine 112 mutated to cysteine according the published nucleosome structures (Luger et al., 1997; Tachiwana et al., 2011). Right panel: Pymol models of the yeast H3 histone fold domain with alanine 111 mutated to cysteines and the Cse4 histone fold domain with leucine 204 according the published H3 nucleosome and (CENP-A^{Cse4}/H4)$_2$ heterotetramer structures (Cho and Harrison, 2011; White et al., 2001). The H3/CENP-A and H3/Cse4 heterodimers are modeled by superimposition of the published homodimer structures. Sulfur atoms are depicted in yellow.

In the crystal structures of human CENP-A nucleosome the two leucines 112 are separated from each other by 4.8 to 5.7 Å (Sekulic et al., 2010; Tachiwana et al., 2011) and in the recently reported crystal structure of the budding yeast CENP-A^{Cse4}/H4 heterotetramer the corresponding leucines 204 are 3.9 – 5.4 Å apart (Figure 2.17). This distance would allow cross-linking if the leucine residues were mutated to cysteines. We designed different CENP-A^{Cse4} constructs for the overexpression of the recombinant proteins (Figure 2.18 and

2.19A). The histone fold domain of CENP-A^{Cse4} (Cse4-D150, starting with aspartate 150, p1487) and the full-length *S. cerevisiae* histone H3 (p1363) were used in the experiments described below. These constructs have a size of 10 kDa and 20 kDa respectively, which facilitates the detection of possible homo- and heterodimers (Figure 2.19B). Since budding yeast histone H3 and CENP-A^{Cse4} do not contain any cysteines, we mutated A111 in histone H3 and L204 in CENP-A^{Cse4} to cysteines (Figure 2.16). To test if a cross-link between two CENP-A^{Cse4} molecules or between CENP-A^{Cse4} and H3 is possible we co-expressed CENP-A^{Cse4}-Cys (with N-terminal 6xHis-tag) and the full-length H3-Cys (with a N-terminal biotinylatable Avitag) in bacteria (Figure 2.19B). Using this approach we could show the spontaneous formation of covalently cross-linked H3 homodimers and CENP-A^{Cse4} homodimers in crude bacterial lysates. Interestingly, we detected some H3/ CENP-A^{Cse4} heterodimers. The dimers were covalently cross-linked since they were detected after denaturing SDS-electrophoresis and could be resolved by the treatment with the reducing agent β-mercaptoethanol (Figure 2.19C). This observation indicates that the H3/ CENP-A^{Cse4} heterodimers are likely to contain the four-helix bundle with the cysteine residues positioned sufficiently close to each other to allow for cross-linking (Figure 2.19C).

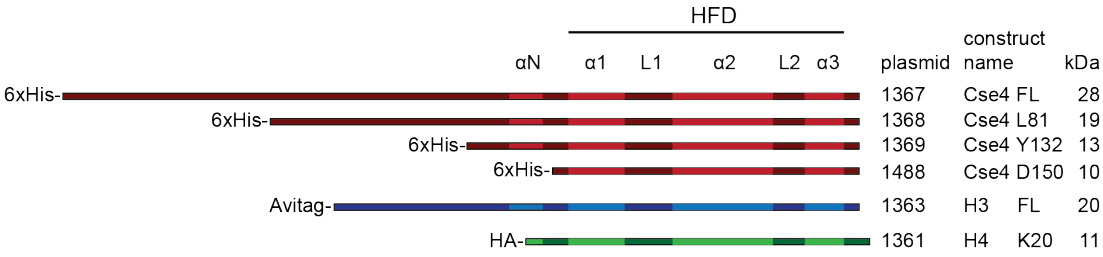

Figure 2.18 Different codon optimized constructs of budding yeast Cse4, histone H3 and H4 for the expression in bacteria

Cse4 constructs are shown in red and contain a N-terminal His6 tag. FL, L81, Y132 and D150 refer to full-length and N-terminal truncations starting with leucine 81, tyrosine 132 and aspartate 150, respectively. Leucine 204 was mutated to a cysteine in these constructs. Histone H3 (blue) had alanine 111 mutated to cysteine and was N-terminally tagged with a biotinylatable Avitag. The histone H4 (green) construct was N-terminally tagged with HA and was an N-terminal truncation starting with lysine 20. The molecular weight of the proteins is indicated in kDa on the right.

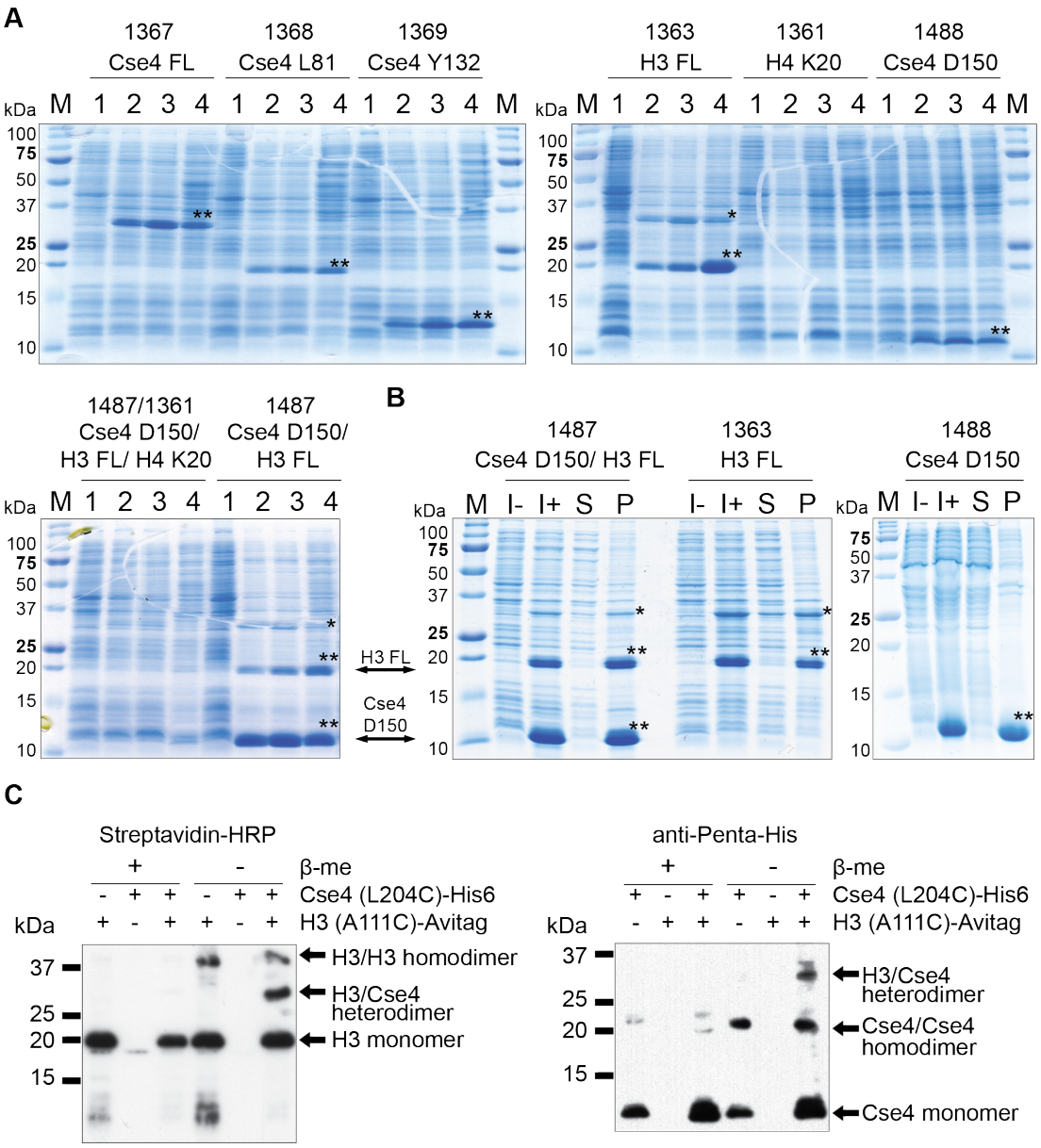

Figure 2.19 Histone H3 and Cse4 dimers can be covalently cross-linked via disulfide bonds between cysteine residues in the four-helix bundle

A) Different L204C mutant constructs of Cse4 tagged with His6, full-length A111C histone H3 tagged with an Avitag and the HA-tagged histone H4 (K20-end) were expressed in bacteria either individually or in different combinations. Samples of crude bacterial lysates before induction (1), 3 h (2), 5 h (3), and 20 h (4) after induction were resolved on a denaturing 15 % SDS-PAGE and stained with Coomassie blue. Two asterisks (**) indicate bands of interest; one asterisk (*) indicates the protein BirA, which was co-expressed to allow the biotinylation of the Avitag on histone H3. B) The constructs 6xHis-Cse4-D150(L204) and Avitag-H3(A111C) were either expressed in combination (1487) or separately (1363 and 1488). Samples were taken before induction (I-), after induction (I+), supernatant (S) and the pellet (P) after cell lysis and centrifugation, resolved on a denaturing 15 % SDS-PAGE, and stained with Coomassie blue. Two asterisks (**) indicate bands of interest; one asterisk (*) indicates BirA. C) Crude bacterial lysates prepared from (B) were separated on 15 % SDS-PAGE and analyzed by Western blot with Streptavidin-HRP recognizing histone H3 tagged with Avitag and anti-Penta-His antibody recognizing Cse4 tagged with His6. H3/H3 homodimers, Cse4/Cse4 homodimers and H3/Cse4 heterodimers are indicated (Lochmann and Ivanov, 2012).

We reasoned that if CENP-A^{Cse4}-Cys and H3-Cys could be cross-linked in vivo it would be a strong evidence for the formation of a heterotypic CENP-A^{Cse4}/H3 octamer. Since budding yeast histone H3 and CENP-A^{Cse4} do not contain any cysteines, we constructed the mutant strains where A111 and L204 (Figure 2.16) were mutated to cysteines. We observed cross-linked H3-Cys homodimers in crude lysates and in isolated chromatin treated with an oxidizing agent 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB, Ellman's reagent) (Figure 2.20A), which was reported to facilitate the formation of intermolecular disulfide bonds between H3 histones in chicken nucleosomes (Bode, 1979) (Figure 2.21A). Furthermore, it was possible to cross-link H3-Cys histones using the cysteine-specific cross-linkers bBBr (Dibromobimane) and BMOE (Bismaleimidoethane), which can cross-link thiol groups that are separated by 5 Å and 8 Å, respectively (Green et al., 2001) (Figure 2.20B and C). However, we could not detect a reproducible cross-link, either between two CENP-A^{Cse4}-Cys molecules or between CENP-A^{Cse4}-Cys and H3-Cys using these cross-linking reagents (Figure 2.21B).

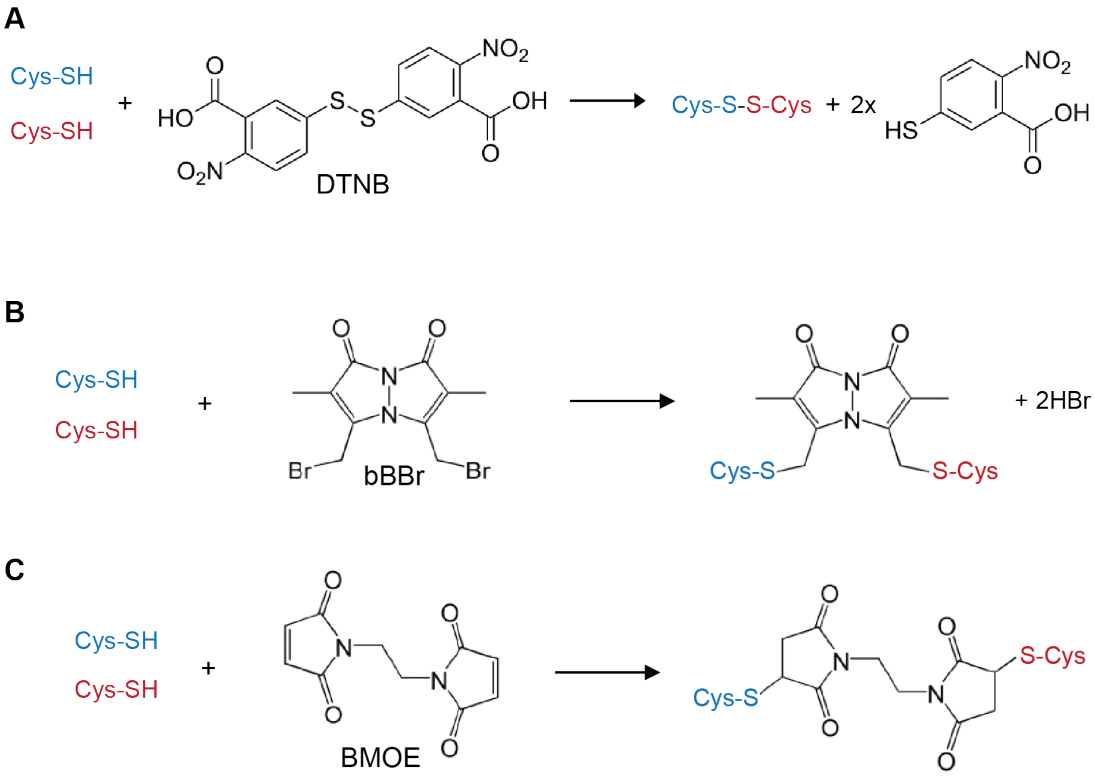

Figure 2.20 Cross-linking reactions of the three reagents employed for the nucleosomal cross-link
A) The zero-distance cross-linker DTNB facilitates the formation of disulfide bonds between proximal thiol groups. B) bBBr and C) BMOE can bridge thiol groups separated by 5 Å and 8 Å, respectively.

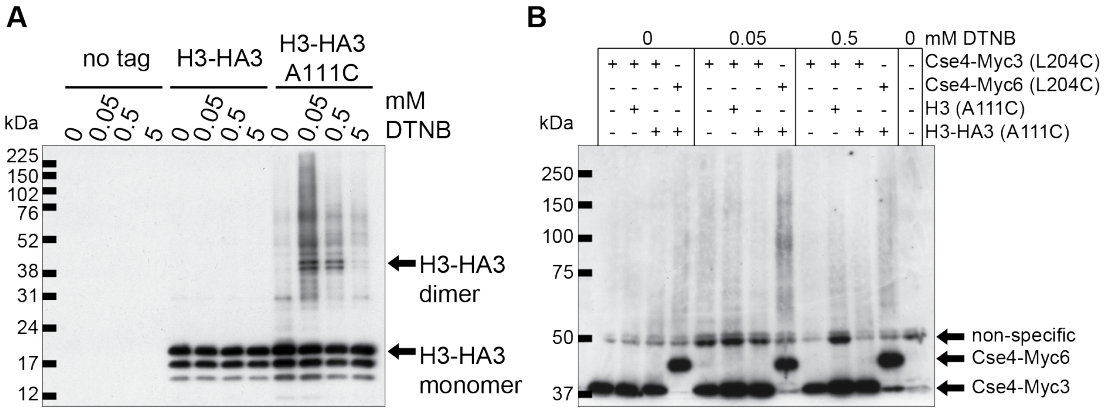

Figure 2.21 Cysteine-containing versions of histone H3 but not Cse4 can be cross-linked on chromatin ex vivo

Chromatin pellets were treated with DTNB to facilitate the disulfide bond formation between the cysteine side chains. Proteins were then eluted with SDS-PAGE loading buffer without β-mercaptoethanol and separated on a 15 % and a 10 % SDS-PAGE, respectively. Western blots were analyzed with anti-HA antibody recognizing tagged H3 (A) or anti-Myc antibody recognizing tagged Cse4 (B). The strains were 1021 (wt), 1266 (H3-HA3), 1268 (H3-HA3 (A111C)) 1924 (Cse4-Myc3 (L204C)), 1949 (Cse4-Myc3 (L204C) H3 (A111C)), 1953 (Cse4-Myc3 (L204C) H3-HA3 (A111C)), and 1955 (Cse4-Myc6 (L204C) H3-HA3 (A111C)) (Lochmann and Ivanov, 2012).

At this moment we have no evidence for the existence of a heterotypic octamer at budding yeast centromeres in vivo. We cannot rule out that the heterotypic nucleosome has a very unusual structure compared to the canonical H3-H3 nucleosome (Luger et al., 1997) or to the human CENP-A-CENP-A octamer (Sekulic et al., 2010; Tachiwana et al., 2011). It is possible that this structure does not allow the cross-link between the two cysteine residues. It has not been reported whether the cysteine residues can be cross-linked in a fully assembled CENP-A/CENP-A or CENP-A/H3 octamer.

2.11 Histone H3 and CENP-A^{Cse4} are not occupying discrete sub-regions within the centromeric DNA

An alternative to the octamer model is the hemisome model, which proposes a tetramer consisting of CENP-A^{Cse4}, histone H4, H2A, and H2B (Dalal et al., 2007a; Dalal et al., 2007b; Furuyama and Henikoff, 2009). Refinement of this model to suggest that there is either a canonical nucleosome or possibly a histone H3-containing hemisome in direct proximity to a CENP-A^{Cse4} hemisome could explain the detection of histone H3 at the centromeric DNA. According to the recently reported structure, 121 bp of DNA are wrapped around the human

CENP-A-containing octamer assembled in vitro (Tachiwana et al., 2011), whereas a conventional nucleosome organizes 147 bp of DNA. Hence, 208 bp of DNA are required to accommodate a CENP-A^{Cse4} hemisome (61 bp) and a conventional nucleosome without any linker in-between. This ensemble would fit with the size of our excised centromeric DNA fragment of 214 bp. An important and verifiable prediction of this model is that CENP-A^{Cse4} and histone H3 are integrated into separate structures, which can be potentially mapped to different sub-regions within the centromeric DNA.

The budding yeast centromere is defined by a 125 bp sequence (Cottarel et al., 1989) consisting of three elements. CDEI is a 8 bp palindrome, CDEII is 78-86 bp long and is composed of 87-98 % A/T, and CDEIII is a highly conserved 25 bp sequence, which binds the CBF3 protein complex (Hegemann and Fleig, 1993). We examined whether CENP-A^{Cse4} and histone H3 associate with separate elements within the centromeric DNA. It was previously reported that *CENP-A^{CSE4}* interacts genetically with CDEI and CDEII but not with CDEIII (Keith and Fitzgerald-Hayes, 2000) suggesting that the CENP-A^{Cse4}-containing nucleosome is located upstream of the CDEII/CDEIII boundary. Since we can cut the minichromosome between CDEII and CDEIII (Figure 2.3A) we hoped to gain further insights in the exact localization of CENP-A^{Cse4} with regard to the centromeric DNA by employing our ChIP method.

We designed a minichromosome with BglII restriction sites to excise a CDEI/II fragment of 139 bp. The analysis of the Southern blot after the ChIP experiment without cross-linking with CENP-A^{Cse4}-HA and H3-HA respectively revealed that the CDEI/II-less fragment is not co-immunoprecipitated with CENP-A^{Cse4}-HA and that CENP-A^{Cse4} mainly localizes to the CDEI/II-containing fragment. As expected H3-HA associates with both the CDEI/II-less and the CDEI/II-containing fragment (Figure 2.22A). We have constructed a minichromosome with BglII restriction sites to excise a CDEIII fragment of 75 bp. However, the size difference of 75 bp between the CDEIII-less and the CDEIII-containing fragments was too small for the detection of two distinct bands by Southern blot. Therefore we generated a minichromosome with a BglII restriction site between CDEII and CDEIII and a BglII restriction site outside of the centromeric DNA, in the *ARS1* sequence. A restriction digest of this

minichromosome generated larger CDEI/II- and CDEIII-containing fragments that could be distinguished by Southern blot. We could co-immunoprecipitate CENP-A^{Cse4}-HA6 with both the CDEI/II- and the CDEIII-containing fragments (Figure 2.22B), implying that the centromeric nucleosome spans the boundary between CDEII and CDEIII. The interaction with the CDEIII fragment appeared less stable, indicating that the CENP-A^{Cse4}-containing nucleosome interacts mostly with the CDEI/CDEII region of the centromeric DNA. An important consequence from this observation is that in our ChIP experiments the CENP-A^{Cse4}-containing nucleosome (or hemisome) is not displaced from the centromeric DNA to the edge of the 214 bp fragment (see also Figure 2.3C and 2.5).

To further map histone H3 and CENP-A^{Cse4} on the centromeric DNA we next excised a 139 bp fragment from a position 50 bp upstream of CDEI until the CDEII/CDEIII boundary. After cross-linking, this fragment could be co-immunoprecipitated with both histone H3-HA and CENP-A^{Cse4}-HA using anti-HA antibodies (Figure 2.23A). The detection of a fragment containing CDEIII and 50 bp of DNA downstream of the centromeric DNA with a double DIG labeled LNA probe was not feasible, therefore we monitored the localization of histone H3 and CENP-A^{Cse4} to the CDEI/II and the CDEIII fragments by employing the ChIP/qPCR method. Both fragments containing the CDEI/II region with 50 bp upstream and the fragment containing the CDEIII region with 50 bp downstream could be co-immunoprecipitated with HA-tagged versions of CENP-A^{Cse4} and histone H3 with and without cross-linking (Figure 2.23B). Therefore histone H3 and CENP-A^{Cse4} appear to be inseparable when associated with the centromeric DNA. This observation implies that CENP-A^{Cse4} and histone H3 are likely to be a part of one and the same structure. It is important to note that since CENP-A^{Cse4} can tether CDEII and CDEIII fragments together (Figure 2.22B), the co-immunoprecipitation of the small CDEI/II and CDEIII fragments with histone H3 could be due to the small CDE-containing fragments maintaining their association with the large CDE-less fragment of the minichromosome throughout our co-immunoprecipitation method. However, no such tethering was observed when the complete 214 bp CEN DNA containing fragment was excised from the minichromosome and immunoprecipitated

(Figures 2.3C). In addition, no tethering was observed between a CEN-containing 930 bp fragment comprising the region from *ARS1* sequence until 50 bp downstream of CDEIII and the CEN-less fragment (Figure 2.5)

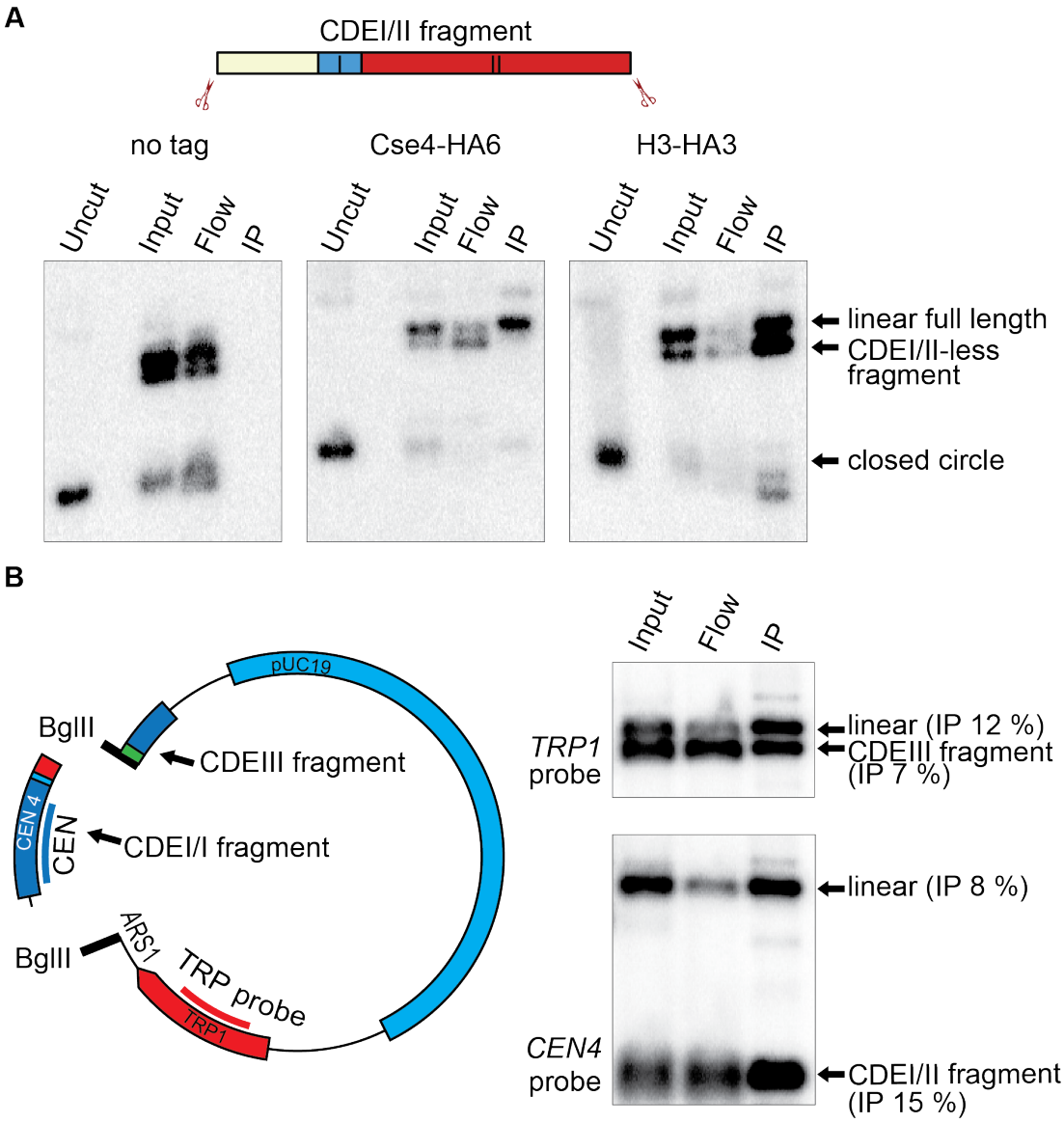

Figure 2.22 Cse4 straddles the CDEII/III boundary
A) Cse4 is mainly associated with the CDEI/II-containing fragment. BglII-treated chromatin of strains carrying the minichromosome p1031 with BglII restriction sites 50 bp upstream of CDEI and between CDEII and III was immunoprecipitated with anti-HA antibody. The strains were 1021 (wt), 1498 (Cse4-HA6), and 1407 (H3-HA3). The DNA was separated on a 0.8 % agarose gel and the Southern blot was analyzed with a ^{32}P labeled *TRP1* probe. B) The Cse4 nucleosome straddles the boundary between CDEII and CDEIII. Left: A Map of the minichromosome p1173 utilized in the experiment. The construct contains 850 bp of pericentromeric sequence of chromosome IV, a *TRP1* marker, the *ARS1* and pUC19 sequence, and has a size of 4.5 kb. There are two BglII sites: between CDEII and CDEIII in the CEN and in the *ARS1*, encompassing 860 bp. Right: BglII-treated chromatin of a strain 1498 (Cse4-HA6) carrying the minichromosome shown to the left was immunoprecipitated with anti-HA antibodies. The DNA was eluted off the beads and separated on a 1 % agarose gel. The Southern blot was analyzed with a ^{32}P labeled probe for the pericentric CEN4 sequence (to detect CDEI/II

containing fragments) and a ^{32}P labeled probe for the *TRP1* gene (to detect CDEIII containing fragments) (Lochmann and Ivanov, 2012).

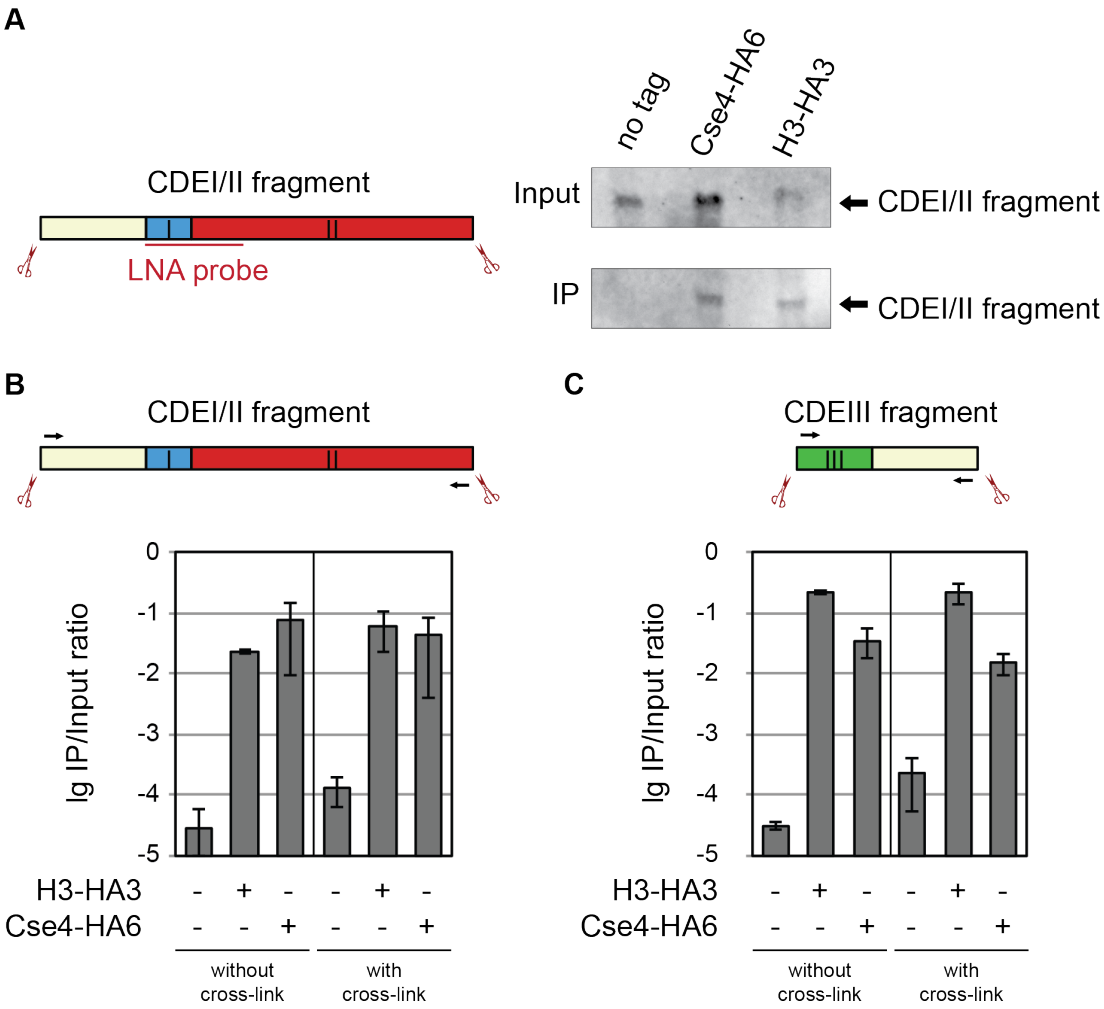

Figure 2.23 Cse4 and histone H3 association with CDEI/II and CDEIII
A) Both Cse4 and histone H3 are associated with the CDEI/II fragment. Left: A scheme of the CDEI/II fragment excised from the minichromosome p1031. The double-DIG labeled LNA probe for CDEI/II is indicated. Right: BglII-treated chromatin of the strains 1498 (Cse4-HA6) and 1407 (H3-HA3) carrying the minichromosome p1031 with BglII sites between CDEII and CDEIII and 50 bp upstream of CDEI was cross-linked with formaldehyde and immunoprecipitated with anti-HA antibodies. The DNA was eluted off the beads and resolved on a 6 % denaturing TBE polyacrylamide gel. The Southern blot was analyzed with a double-DIG labeled LNA probe for CDEI/II. B and C) Both the CDEI/II and the CDEIII fragments can be co-immunoprecipitated with Cse4 and H3. Strains 1021 (wt), 1407 (H3-HA3), and 1498 (Cse4-HA6) carried the minichromosome where either the CDEI/II (p1031) (B) or the CDEIII fragment (p1032) (C) was flanked with BglII sites. BglII-treated chromatin was either not cross-linked or cross-linked with formaldehyde and immunoprecipitated with anti-HA antibody. The immunoprecipitated DNA was purified, size fractionated, and subjected to qPCR analysis. The bar graphs represent the average values from several independent experiments with SDs (Lochmann and Ivanov, 2012).

3 Discussion

3.1 Our findings in the context of the proposed models for the centromeric nucleosome

Three different models of the centromeric CENP-A nucleosome are proposed in the literature. The first model is the so-called octamer model (Figure 1.8B), where CENP-A replaces both histone H3 molecules in the centromeric nucleosome. While octameric yeast CENP-A^{Cse4} nucleosomes (Dechassa et al., 2011; Kingston et al., 2011) or human CENP-A nucleosomes (Tachiwana et al., 2011) can be assembled in vitro, it is unknown whether only one or both copies of H3 are replaced in vivo. There is evidence for and against either of these possibilities from different organisms. In HeLa cells, CENP-A is still associated with histone H3 after its release from chromatin by micrococcal nuclease digestion and the treatment with high salt resulting in dissociation of the DNA, histones H2A, and H2B. These results are indicating the existence of heterotypic tetramers with two histones H4, one H3 and one CENP-A (Foltz et al., 2006). Conversely, when chromatin from *Drosophila* S2 and Kc cells is digested with micrococcal nuclease and CENP-A^{CID} is immunoprecipitated, histone H3 does not co-purify with CENP-A^{CID} (Blower et al., 2002). Moreover, it was reported recently that *Drosophila melanogaster* CENP-A^{CID} forms homodimers in vivo. The CENP-A^{CID} homodimers are unexpectedly very salt-sensitive but mononucleosomes could be cross-linked via cysteines in the four-helix bundle after prolonged incubation (Zhang et al., 2012). It is important to note that CENP-A^{CID} contains two cysteine residues, one in the middle of the alpha two helix (C184) and one at the C-terminal end of the alpha three helix (C219) (Figure 1.5) whereas *Drosophila* histone H3, which was used as the positive control for the cross-linking experiment, contains a single cysteine residue at the C-terminal end of the alpha two helix (C111). It was not established which of the two cysteine residues of CENP-A^{CID} was cross-linked. Furthermore, the possible formation of H3-CENP-A^{CID} heterodimers in addition to CENP-A^{CID} homodimers was not addressed by the authors (Zhang et al., 2012) and it remains possible that diverse forms of CENP-A^{CID} nucleosomes are simultaneously present at the regional centromeres of *Drosophila* and possibly

other higher eukaryotes. However, the cross-linking of histone H3 and CENP-A^{CID} is a very unlikely event in this experimental setup since the cysteine residues of histone H3 and CENP-A^{CID} are too far apart from each other.

In my thesis, I report the association of both histone H3 and CENP-A^{Cse4} with a centromeric DNA fragment of only 214 bp in budding yeast. Our data suggests a very intimate spatial association between the conventional histone H3 and centromeric CENP-A^{Cse4}, which is incompatible with a homotypic CENP-A^{Cse4} octamer. This intimate spatial association cannot be explained if the CENP-A^{Cse4}-containing centromeric nucleosome is separated from the adjacent canonical H3 nucleosomes by spacer DNA as was suggested recently (Cole et al., 2011) but rather implies that histone H3 and CENP-A^{Cse4} co-occupy the centromeric DNA fragment of only 214 bp in length. We favor the heterotypic octamer model with one copy each of CENP-A^{Cse4} and histone H3 and two copies each of the histones H4, H2A, and H2B (Figure 3.1 and 1.9). There is evidence suggesting that this octamer might be resistant to cysteine cross-linking because of a reduced stability of the four-helix bundle as observed by the *Drosophila* CENP-A^{CID} (Zhang et al., 2012).

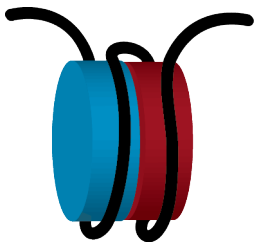

Figure 3.1 Model of the heterotypic CENP-A^{Cse4}/H3 nucleosome
The heterotetramer consisting of the histone H3, H4, H2A, and H2B is depicted in blue and the heterotetramer containing CENP-A^{Cse4} instead of histone H3 is depicted in red. The heterotypic octamer consists of a H3 and a CENP-A^{Cse4} containing heterotetramers and probably organizes 134 bp of DNA (black) in a left-handed manner, 61bp for the CENP-A^{Cse4} - and 73 bp for the H3-containing heterotetramer (Lochmann and Ivanov, 2012).

The hexamer model (Figure 1.10B) postulates that, in budding yeast, the non-histone protein Scm3 replaces H2A and H2B and the centromeric nucleosome is comprised of two copies each of Scm3, CENP-A^{Cse4} and H4 (Mizuguchi et al., 2007; Xiao et al., 2011). Although it was originally suggested that the Scm3 dimer forms an integral part of the centromeric hexasome (Mizuguchi et al., 2007), the recent structures of budding yeast Scm3 associated

with the CENP-A^{Cse4}/H4 heterodimer (Cho and Harrison, 2011; Zhou et al., 2011) and human HJURP in complex with the CENP-A/H4 heterodimer (Bassett et al., 2012; Hu et al., 2011) revealed that DNA binding, as well as the formation of the (CENP-A^{Cse4}/H4)$_2$ heterotetramer, is incompatible with the simultaneous binding of Scm3. Furthermore, in vitro experiments showed that the association of Scm3 with the reconstituted (CENP-A^{Cse4}/H4)$_2$ nucleosome-like particles depends on a DNA binding domain within Scm3 (Xiao et al., 2011). Our findings are in agreement with the view that Scm3 does not constitute an integral part of the centromeric nucleosome. We could co-immunoprecipitate minichromosomes and its fragments with CENP-A^{Cse4}, H4, H2A, H2B and H3 but not with Scm3 under our experimental conditions, which did not include cross-linking. Most likely, Scm3 dissociated from the centromere in yeast cell lysate.

Finally, the hemisome model (Figure 1.10A) suggests that the centromeric nucleosome is a tetramer consisting of CENP-A, H4, H2A and H2B histones (Dalal et al., 2007a; Dalal et al., 2007b; Dimitriadis et al., 2010; Furuyama and Henikoff, 2009). According to this model, the CENP-A^{Cse4} hemisome is located mostly at CDEII (Krassovsky et al., 2012) and is expected to occupy approximately 60 bp of DNA (Tachiwana et al., 2011). In our experimental setup the hemisome would leave approximately 77 bp on each side of the excised 214 bp fragment available to accommodate the histone H3-containing nucleosome(s). We can speculate that a hemisome with CENP-A^{Cse4} might, for example, be incorporated into a DNA loop between the two halves of an H3-containing octamer (Figure 3.2).

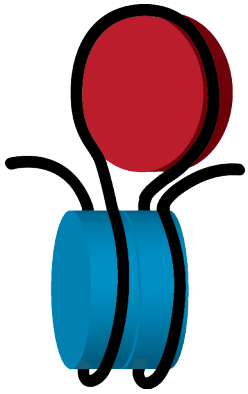

Figure 3.2 A CENP-A^{Cse4} hemisome incorporated into a loop of a canonical histone H3 nucleosome
The canonical octamer, consisting of two copies each of the histones H3, H4, H2A, and H2B, is depicted in blue. The hemisome, containing CENP-A^{Cse4} instead of histone H3, as well as H4, H2A,

and H2B is depicted in red. This model organizes 208 bp of DNA, 147 bp are wrapped around the canonical H3 nucleosome in a left-handed manner and 61 bp are organized by the CENP-A^{Cse4} hemisome in a right-handed manner (Lochmann and Ivanov, 2012).

This model could explain the tripartite organization of the budding yeast centromere that was observed in a recently published micrococcal nuclease protection pattern (Krassovsky et al., 2012). Alternatively, it is technically possible that 77 bp upstream and downstream of the hypothetical centromeric hemisome are wrapped around halves of the two neighboring canonical nucleosomes (Figure 3.3A). However, in this case the excised 214 bp centromeric DNA fragment is expected to be tethered to the CEN-less fragment of the minichromosome. Such tethering would result in co-immunoprecipitation of both CEN-containing and CEN-less fragments of the minichromosomes with CENP-A^{Cse4}, which was not observed in our assays and therefore can be excluded (Figure 2.3C and 2.5). The model of a CENP-A^{Cse4} containing hemisome flanked by one histone H3 nucleosome (Figure 3.3B) or the existence of a neighboring histone H3-containing hemisome next to a CENP-A^{Cse4} hemisome (Figure 3.3C) can also be excluded since we did not observe the localization of histone H3 and CENP-A^{Cse4} to separate subregions within the centromere (Figure 2.22B and 2.23).

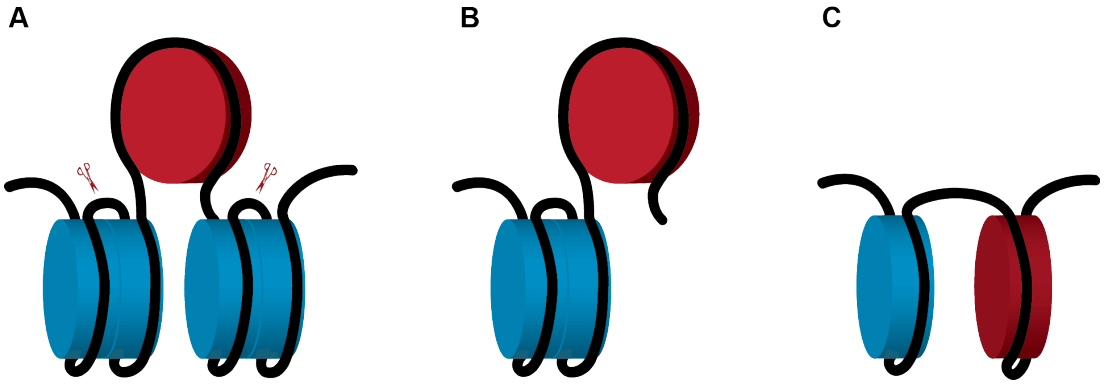

Figure 3.3 Models that combine histone H3 and CENP-A^{Cse4} at the centromere but are not supported by our findings

A) A CENP-A^{Cse4} hemisome (red) that is flanked by two canonical nucleosomes (blue). The scissors indicate the BglII restriction sites. This model predicts the tethering of the CEN-less fragment of the minichromosome to CENP-A^{Cse4}, which was not observed in our assay (Figure 2.3C and 2.5) (Lochmann and Ivanov, 2012). B) A CENP-A^{Cse4} hemisome (red) and one flanking canonical nucleosome (blue) organize 208 bp of DNA (black) without any linker in-between. Should this model be true, histone H3 and CENP-A^{Cse4} would be confined to the distinct sub-regions of the centromeric DNA (Figure 2.22B and 2.23). The same applies to model C), where a CENP-A^{Cse4} hemisome and a histone H3 hemisome organize 134 bp of DNA (no linker included) in a left- and right-handed

manner, respectively. We were not able to separate the centromeric DNA into histone H3- and CENP-A^{Cse4} "domains".

Interestingly, very recent data supports the view that only one CENP-A^{Cse4}-EGFP molecule is present per centromere through most of the budding yeast cell cycle by fluorescence correlation spectroscopy (FCS) (Shivaraju et al., 2012). Only during anaphase were two molecules of CENP-A^{Cse4}-EGFP observed per centromere. Furthermore, the authors show that CENP-A^{Cse4}-EGFP is deposited to the centromere not only at S phase, which was shown previously by (Pearson et al., 2004) but also during anaphase. By employing the fluorescence energy transfer (FRET) technique and a sequential ChIP, the authors show that two differently tagged versions of CENP-A^{Cse4} (CENP-A^{Cse4}-EGFP with CENP-A^{Cse4}-mCherry and CENP-A^{Cse4}-Myc12 and FLAG-CENP-A^{Cse4} respectively) are interact with each other exclusively in anaphase (Shivaraju et al., 2012). The authors conclude that the centromeric nucleosome is oscillating between the CENP-A^{Cse4} hemisome and the homotypic CENP-A^{Cse4} nucleosome.

However, there are fundamental problems with the proposed model, which remain unanswered. It was postulated that a CENP-A^{Cse4} hemisome wraps the DNA in a right-handed manner, which would not be possible for the CENP-A^{Cse4}/CENP-A^{Cse4} nucleosome due to structural constrains (Furuyama and Henikoff, 2009). Therefore, the proposed transition from a hemisome to an octameric nucleosome would imply a reversal of the handiness of the DNA wrap. It is reasonable to assume, that the DNA has to be unwound for this purpose. Are there centromere-specific proteins, which might facilitate this transition? Scm3 appears to be well suited for the proposed role. Homotypic CENP-A^{Cse4} nucleosomes that were assembled in vitro in the presence of NAP1 (nucleosome assembly protein 1), induce negative supercoils in closed circular DNA (Shivaraju et al., 2012). However, in the presence of Scm3 the assembled CENP-A^{Cse4} nucleosomes are not clearly inducing either positive or negative supercoils in the closed circular DNA. Could this be a transition state between the CENP-A^{Cse4}/CENP-A^{Cse4} nucleosome and the CENP-A^{Cse4} hemisome? The authors conclude that the supercoils are more positive when compared to the NAP1 assembled nucleosomes and propose a mix between left- and right-handed nucleosome but do not address the exact composition of these different

complexes further (Shivaraju et al., 2012). Interestingly, a very similar assay by (Dechassa et al., 2011) did not reveal a positive supercoiling. It is possible that the observed positive supercoils are induced by CENP-A^{Cse4}/H4/Scm3 complexes or (CENP-A^{Cse4}/H4)$_2$ tetramers associated with DNA. It is interesting to note that the H2A/H2B dimers are less stably associated with the in vitro assembled CENP-A^{Cse4} nucleosome as compared to the canonical nucleosome (Dechassa et al., 2011). For the (H3/H4)$_2$ tetramer it was observed that they can wrap the DNA in a left- and a right-handed manner (Hamiche et al., 1996) and (CENP-A^{Cse4}/H4)$_2$ tetramers might display similar properties. Since the in vitro assay also contains the histones H2A and H2B, homotypic CENP-A^{Cse4} nucleosomes might also be formed, which complicates the interpretation of the supercoiling assay further. However, it was shown that the CENP-A^{Cse4}/H4/Scm3 complex does not induce supercoils into closed circular DNA, only by the addition of the H2A/H2B dimer to the CENP-A^{Cse4}/H4/Scm3 complex supercoils are induced (Shivaraju et al., 2011).

The reported results of (Shivaraju et al., 2012) and their interpretation that the centromeric nucleosome is oscillating between the CENP-A^{Cse4} hemisome and the homotypic CENP-A^{Cse4} nucleosome are very intriguing. Since it remains possible that the observed positive supercoils are induced by (CENP-A^{Cse4}/H4)$_2$ tetramers devoid of the histones H2A and H2B, it remains to be confirmed that if the hemisome exists, whether it really wraps the DNA in a left-handed manner. The discovery of the hemisome could be simply a result of the reduced stability of the four-helix bundle linking two molecules of CENP-A or CENP-A and H3. Considering that in our experiments histone H3 and CENP-A^{Cse4} co-immunoprecipitate with the centromeric DNA when yeast are arrested with alpha factor and nocodazole (Figure 2.8), we propose that histone H3 and CENP-A^{Cse4} co-occupy the centromeric DNA in both G1 and the G2 of the cell cycle and form a heterotypic CENP-A^{Cse4}/H3 nucleosome (Figure 3.4).

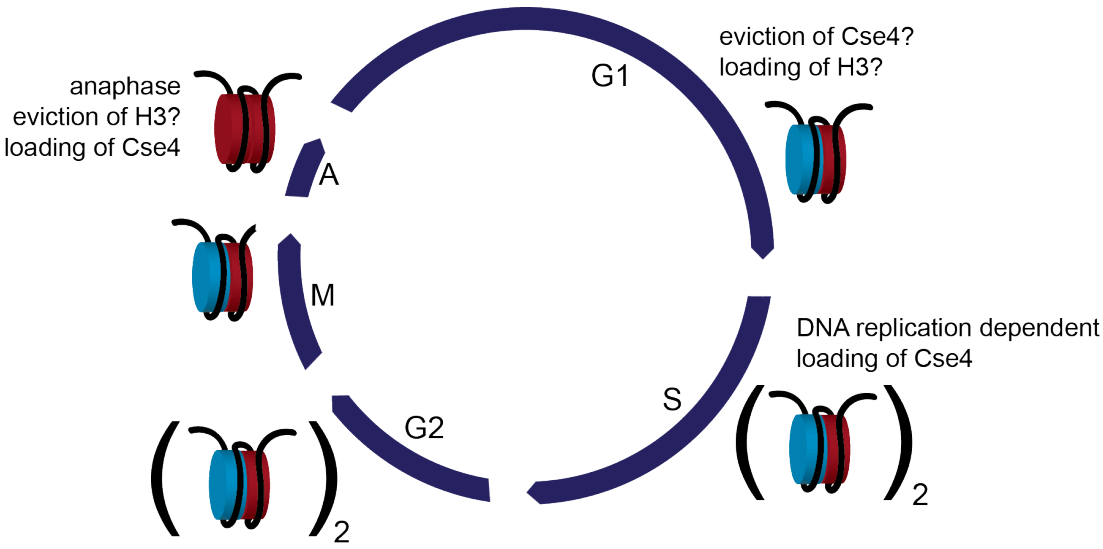

Figure 3.4 Possible structural changes of the centromeric CENP-A^{Cse4} nucleosome during the cell cycle

The heterotypic CENP-A^{Cse4} nucleosome is depicted as in (Figure 3.1) the homotypic CENP-A^{Cse4} nucleosome is shown in red. The scheme is adapted from (Shivaraju et al., 2012; Westhorpe and Straight, 2012) and is modified to accommodate our finding that histone H3 and CENP-A^{Cse4} co-occupy the centromeric DNA. For further explanation see text.

3.2 Are homotypic CENP-A^{Cse4} nucleosomes a result of overexpression?

Our results appear to contradict those of (Camahort et al., 2009). This group could co-immunoprecipitate differentially tagged versions of CENP-A^{Cse4} from budding yeast but did not observe co-immunoprecipitation of tagged CENP-A^{Cse4} and H3. However, one of the tagged versions of CENP-A^{Cse4} was expressed from a plasmid and CENP-A^{Cse4} overexpression was reported to result in its ectopic incorporation genome-wide into octameric nucleosomes that were not observed in the wild type strain (Krassovsky et al., 2012). It remains possible that even in budding yeast there is a degree of heterogeneity in the composition of the centromeric nucleosomes among different chromosomes and that either a homotypic CENP-A^{Cse4}/ CENP-A^{Cse4} octamer or a heterotypic CENP-A^{Cse4}/H3 octamer can provide the essential function.

3.3 The localization of the centromeric nucleosome

It was proposed that the centromeric nucleosome is confined to CDEI and CDEII, since CDEI and CDEII genetically interact with CENP-A^{Cse4} and the CDEIII element is bound to the CBF3 complex. This model implies that the centromeric nucleosome organizes non-centromeric DNA upstream of CDEI since CDEI and CDEII would be too short to accommodate a nucleosome (Keith and Fitzgerald-Hayes, 2000). In our ChIP experiments we observed immunoprecipitation of the CDEIII-containing fragment with CENP-A^{Cse4}. Our data support the notion that the centromeric nucleosome organizes the entire centromeric DNA sequence of ~120 bp (Cole et al., 2011). Moreover, several independent studies suggest that the CENP-A containing octamer organizes 115 to 121 bp of DNA instead of the 147 bp occupied by the conventional nucleosome (Cole et al., 2011; Dechassa et al., 2011; Kingston et al., 2011; Tachiwana et al., 2011). Intriguingly, this corresponds to the length of the centromeric DNA in budding yeast.

In budding yeast, the CDEI and CDEIII elements of the centromeric DNA are conserved among all sixteen chromosomes, whereas the CDEII elements vary in length and sequence but all have an A/T content of more than 90 % (Figure 1.2). It was reported that the high A/T content of CDEII facilitates a natural curvature of the centromeric DNA, which is different among the chromosomes (Bechert et al., 1999). The detected differences in the curving were proposed to be balanced by the differences in length of the CDEII elements, thereby resulting in the uniform structural features of the DNA accommodating a single centromeric nucleosome (Cole et al., 2011). If these assumptions are right and the centromeric nucleosome is a heterotypic octamer as our study suggests, then the CDEI and CDEIII elements would be organized by the H2A/H2B dimers and the CDEII element would mainly interact with the heterotypic CENP-A^{Cse4}/H3/$H4_2$ heterotetramer. As a consequence, the Cbf1 protein and the CBF3 complex would be bound to the CDEI and CDEIII elements, respectively, at the flanks of the nucleosome (Figure 3.5).

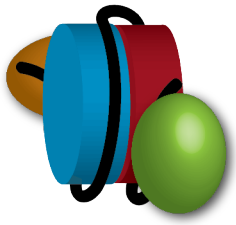

Figure 3.5 Scheme of the heterotypic nucleosome and its association with Cbf1 and CBF3
Shown is the heterotypic CENP-A^{Cse4} nucleosome with the histone H3 and the CENP-A^{Cse4} heterotetramers depicted in blue and red respectively. The nucleosome organizes ~ 120 bp of DNA. The ends represent CDEI and CDEIII elements, which are associated with Cbf1 (green) and the CBF3 complex (orange), respectively.

It is interesting to note that the H2A/H2B dimers are less stably associated with the in vitro assembled CENP-A^{Cse4} nucleosome, compared to the canonical nucleosome (Dechassa et al., 2011). The observed protection of 80 bp of centromeric DNA in budding yeast upon extensive digestion of native chromatin with MNase (Krassovsky et al., 2012) could be a consequence of the nuclease invading into the superhelical regions -6.5 to -3.5 and +3.5 to +6.5, which are normally bound to H2A/H2B. If these regions are clipped, then the size of the central H3/ CENP-A^{Cse4} /H4$_2$-bound fragment would be close to the ~80 bp CDEII fragment observed by (Krassovsky et al., 2012). In addition, two peripheral fragments corresponding to CDEI and CDEIII would be released and possibly protected by Cbf1 and the CBF3 complex. Therefore, it is possible that the MNase protection pattern observed by (Krassovsky et al., 2012) originates from the digestion of the DNA within the centromeric nucleosomal octamer rather than from a hemisome as was proposed by the authors.

3.4 More exotic models of the centromeric nucleosome

Recently two in vivo studies compared CENP-A^{Cse4}-GFP fluorescence to independent standards and discovered 3.5-6.0 (Lawrimore et al., 2011) and even 7.6 (Coffman et al., 2011) CENP-A^{Cse4}-GFP molecules per budding yeast centromere during anaphase. Even more unexpectedly, during a prolonged G1 arrest a more than two-fold decrease of CENP-A^{Cse4}-GFP fluorescence was observed (Coffman et al., 2011). These findings contradict the concept of a single CENP-A^{Cse4} nucleosome at the budding yeast centromere (Furuyama and

Biggins, 2007). It was suggested that the budding yeast centromere is a regional centromere with additional CENP-A^{Cse4} molecules associated with the flanking DNA, similar to the much larger regional centromeres of higher eukaryotes (Lawrimore et al., 2011). However, in our experimental setup we did not detect any CENP-A^{Cse4} associated with the non-centromeric part of the 2.4 kb minichromosome, which is expected to assemble 10 conventional nucleosomes (Figure 2.3C). Therefore, no additional CENP-A^{Cse4} nucleosomes assemble, at least at these relatively short flanking sequences. Our results are in agreement with those of (Shivaraju et al., 2012), who observed only one or two molecules of CENP-A^{Cse4} by fluorescence quantification of CENP-A^{Cse4}-EGFP. Moreover, in a study by (Henikoff and Henikoff, 2012; Krassovsky et al., 2012) no additional CENP-A^{Cse4} nucleosomes were observed in centromere-flanking regions by high-resolution mapping of the yeast genome. It is important to note that the additional CENP-A^{Cse4} molecules at the centromere could be the result of aneuploidy or an inadequate standard used for quantification, as well the mis-incorporation of CENP-A^{Cse4} at ectopic loci, which is observed in strains overexpressing CENP-A^{Cse4} (Krassovsky et al., 2012) and could potentially be caused by tagging CENP-A^{Cse4} with GFP. Alternatively, additional CENP-A^{Cse4} molecules could be associated with the centromeric nucleosome through protein-protein and/or protein-DNA interactions (Figure 3.6).

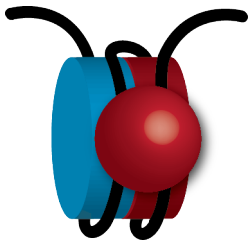

Figure 3.6 Model of the heterotypic CENP-A^{Cse4}/H3 nucleosome with additional CENP-A^{Cse4} bound to it

The same as in Figure 3.1 with additional CENP-A^{Cse4} bound to the heterotypic octamer (Lochmann and Ivanov, 2012).

In the latter scenario, the centromeric nucleosome can be a CENP-A^{Cse4}/H3 heterotypic octamer to which more CENP-A^{Cse4} molecules are bound. Intriguingly, when (CENP-A^{Cse4}/H4)$_2$ heterotetramers were reconstituted in vitro in the presence of Scm3 into nucleosome-like particles on 207 bp-long

high affinity nucleosome positioning DNA sequence, high molecular weight complexes possibly representing additional CENP-A^{Cse4}/H4 in loose association with the CENP-A^{Cse4}/H4/DNA complex were detected (Dechassa et al., 2011). Similar complexes were reported to be assembled in vitro on a 148 bp CEN3 DNA (Xiao et al., 2011).

3.5 Why was histone H3 not discovered at the budding yeast centromere before?

It is more than a decade now since it was proposed that H3 is replaced by the histone variant CENP-A^{Cse4} (Meluh et al., 1998). Our results appear to contradict this well-established dogma. Why was the co-localization of CENP-A^{Cse4} and histone H3 not noticed before? We can propose the following explanation. In most publications reporting ChIP experiments at the budding yeast centromere, the absolute efficiency of ChIP of the centromeric DNA with H3 and CENP-A^{Cse4} is very similar and typically in the range of 1 % (Camahort et al., 2009; Mizuguchi et al., 2007). The claim that only CENP-A^{Cse4} is associated with the centromeric DNA is then based on the observation that non-centromeric DNA is co-immunoprecipitated with histone H3 (and all other histones) at about 5 to 10-fold higher rate than the centromeric DNA while almost no non-centromeric DNA is found associated with CENP-A^{Cse4} (Figure 3.7).

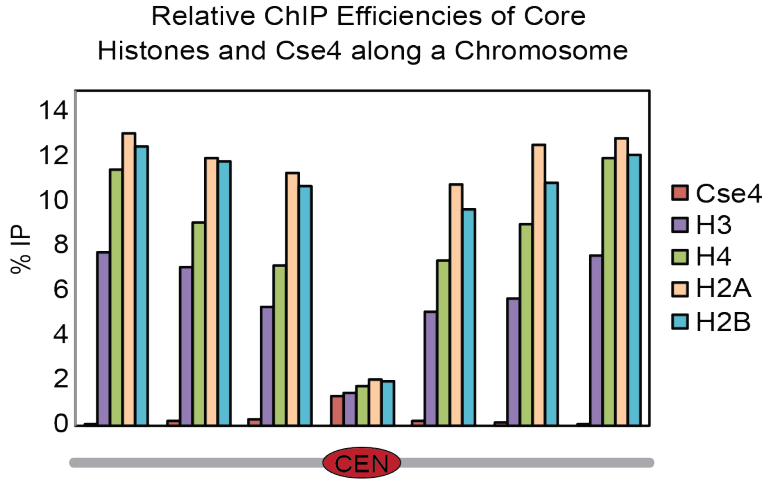

Figure 3.7 ChIP efficiencies of CENP-A^{Cse4} and canonical histones at different locations along a chromosome

Typical ChIP efficiencies are plotted according to the results from previous reports. The efficiency of ChIP at the centromere is usually abound 1 % for canonical histones and CENP-A^{Cse4}. Sequences along the chromosome arm are co-immunoprecipitated with canonical histones with a 5-10 fold higher efficiency, whereas CENP-A^{Cse4} co-immunoprecipitates with a 5-10 fold lower efficiency (Lochmann and Ivanov, 2012).

We propose that if the centromeric DNA were generally difficult to co-immunoprecipitate, for example due to cross-linking of the large number of kinetochore proteins during the in vivo cross-linking, this would explain the reduced efficiency of histone H3 ChIP and the other histones at the centromere compared to the chromosomal arms. In contrast, under our experimental conditions, i.e. either in the absence of cross-linking or with the cross-linker added immediately after cell lysis, the efficiencies of the centromeric DNA co-immunoprecipitation with histone H3 and CENP-A^{Cse4} are indeed very similar.

3.6 The 'problem' of nucleosome sliding

By modifying our experimental protocol we were able to rule out potential artifacts stemming from both cross-linking and nucleosomal sliding (Figure 3.8). With our multi-method approach we excluded the possibility that our findings are the result of a CENP-A^{Cse4} nucleosome sliding out of the centromeric DNA and a H3 nucleosome sliding in. This scenario would involve the rearrangement of at least three nucleosomes. One flanking canonical nucleosome has to move out to vacate the space for the CENP-A^{Cse4} nucleosome, the CENP-A^{Cse4} nucleosome slides out of the centromeric DNA onto the flanking DNA, and a second canonical nucleosome slides onto the centromere. This sliding of the CENP-A^{Cse4} nucleosome onto the non-centromeric part of the minichromosome would result in the co-immunoprecipitation of the CEN-less fragment with CENP-A^{Cse4}, which was not observed in our assays even in the absence of any cross-link (Figure 2.3C and 2.5).

Another possibility we can exclude is that the CENP-A^{Cse4} nucleosome was disengaged from the centromeric DNA and a flanking conventional nucleosome slid into its place. If CENP-A^{Cse4} had the tendency to be released from the DNA during our experimental procedure, this would reduce the efficiency of immunoprecipitation. However, even after prolonged incubations

without cross-link, nearly 100 % of the minichromosomes could be co-immunoprecipitated with CENP-A^{Cse4} (Figure 2.2A). While the efficiency of co-immunoprecipitation of the smaller centromeric DNA fragments with CENP-A^{Cse4} was less than that of an intact minichromosome, the fact that the fragment was excised within 5 minutes makes it very unlikely that a local re-arrangement was taking place in those assays.

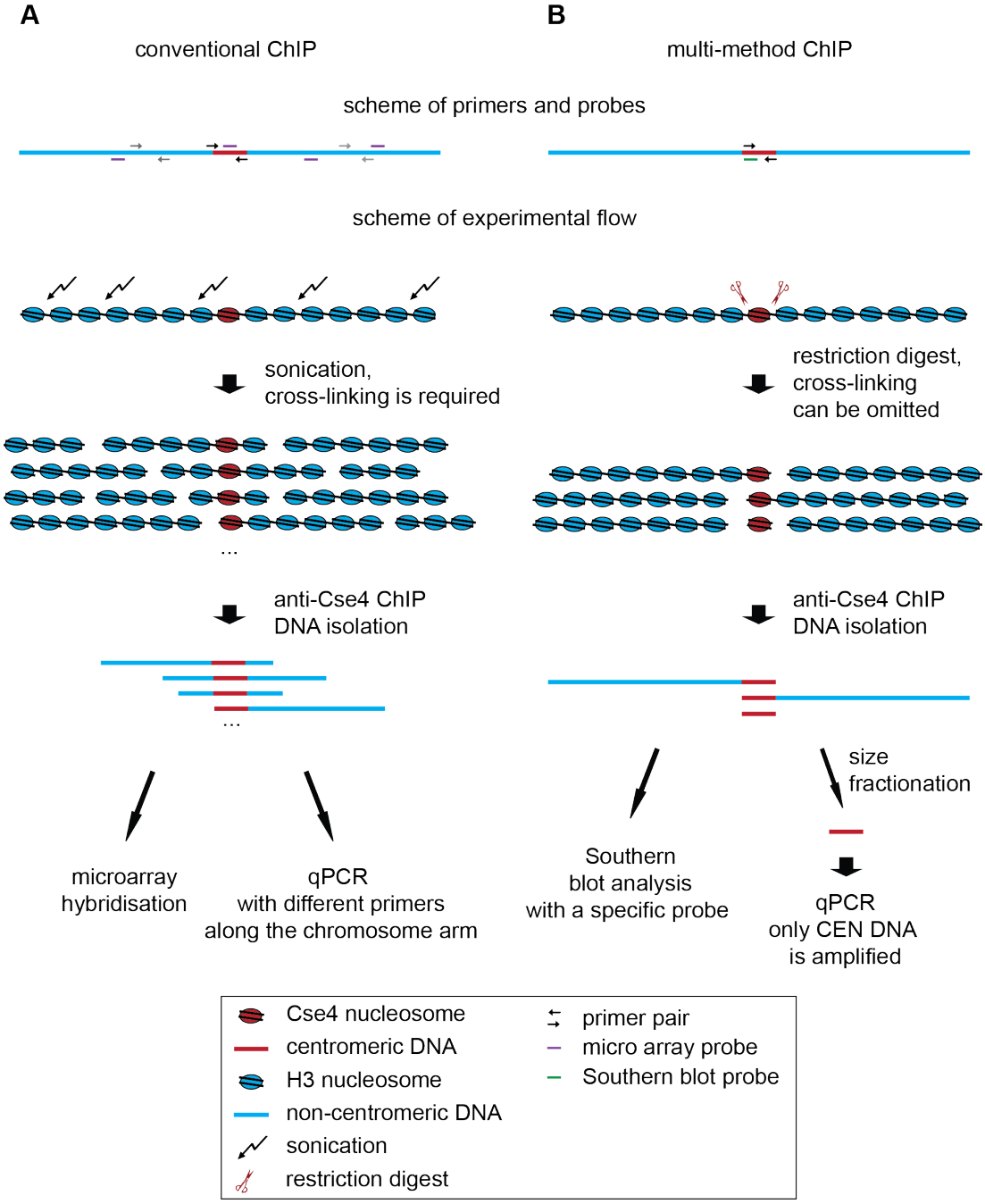

Figure 3.8 Comparison of the conventional ChIP and our multi-method ChIP approach
A) A scheme of the conventional ChIP. The cells are usually cross-linked and the chromatin is fragmented by sonication. After ChIP the isolated DNA is analyzed by microarray hybridization or

PCR methods. The information about the exact size of the co-immunoprecipitated DNA is lost during this experimental approach. B) Scheme of our novel multi-method ChIP. The chromatin is digested at specific sites with restriction enzyme and can be cross-linked if necessary. The limited number of fragments can be either specifically analyzed and identified by Southern blot or the small centromeric DNA fragment can be further purified by size fractionation and analyzed in a highly sensitive qPCR assay to compare ChIP efficiencies of different proteins.

3.7 The cross-linking of nucleosomes via cysteine residues in the four-helix bundle

If the centromeric nucleosome is indeed a heterotypic octamer, it should be possible to cross-link histones H3 and CENP-A^{Cse4} via proximal cysteines in the four-helix bundle. Our inability to obtain this cross-link is puzzling. The formation of a disulfide bond between the two naturally occurring cysteines in H3 of higher eukaryotes under oxidizing conditions (in the presence of a zero-distance sulfhydryl group oxidation agent) was observed by several research groups earlier (Bode, 1979; Gould et al., 1980) and most recently in (Zhang et al., 2012). It was also commented upon in the report of the first crystal structure of the human nucleosome (Luger et al., 1997). In the crystal structure of the conventional nucleosome the sulfhydryl groups are separated by 6.2 Å, which was considered too far for the disulfide bond to form without the substantial distortion of the $(H3/H4)_2$ heterotetramer. The authors mutated the cysteine residue in the human histone H3 to alanine in their study to avoid the disturbance of the structure (Luger et al., 1997). The disulfide bonds between histone H3 cysteines can be formed even in the chromatin isolated from budding yeast when a cysteine is introduced in the corresponding position by mutagenesis (Figure 2.21A). The distances between the corresponding residues of human CENP-A and yeast CENP-A^{Cse4} in CENP-A nucleosomes are even shorter than those between histone H3 cysteines in a human canonical nucleosome based on the available structures (Cho and Harrison, 2011; Tachiwana et al., 2011). We cannot rule out, of course, that CENP-A^{Cse4} octamers are more rigid and will not allow the distortion that was stipulated to be necessary for the formation of the disulfide bonds in the context of the canonical nucleosome. Alternatively, the heterotypic octamer has a unique structure, which is significantly different from the canonical nucleosome and the CENP-A nucleosome. Clearly, it would be important to isolate the centromeric

nucleosomes from budding yeast in significant quantity and purity and characterize them structurally. However, this project is very challenging and is likely to require years of work.

3.8 Conclusion

We show for the first time that histone H3 is not excluded from the centromeric DNA in budding yeast. At this time we can only speculate about the function of histone H3 at the budding yeast point centromere. It is possible that the presence of a heterotypic nucleosome or a canonical nucleosome and a hemisome, one with H3 and one with CENP-A^{Cse4}, provides a structural asymmetry that could form the basis for two separate surfaces, one facing the sister centromere and another providing the foundation for the recruitment of kinetochore components and building the attachment site for the spindle microtubule. Indeed the regional centromeres of higher eukaryotes show a very similar structure. Centromeric chromatin with histone H3 nucleosomes pointing towards the sister chromatid and CENP-A chromatin facing the microtubule attachment site.

With the development of our multi-method ChIP (Figure 3.8B) approach we were able to circumvent the obstacles of the conventional ChIP protocols and provide an elegant tool that can be used in the future to address the localization of various sequence-specific proteins or the composition of protein complexes at a specific DNA sequence.

4 Materials and Methods

4.1 Molecular biology techniques

4.1.1 Polymerase chain reaction

PCR reactions were performed in a BioRad DNA Engine PTC-200 using Fermentas and PeqLab enzymes and buffers, following the manufacturer's guidelines.

4.1.2 Site directed mutagenesis of plasmid DNA

Site directed mutagenesis of plasmid DNA was performed using the Quikchange Site-Directed Mutagenesis Kit from Stratagene. The primers were created according to the manufacturer's guidelines.

4.1.3 Transformation of *E.coli*

Chemically competent E.coli cells were transformed by the addition of at least 1 ng of plasmid DNA to 100 µl of competent bacteria ($CaCl_2$ method) on ice. After a 30 min incubation on ice, the cells were heat-shocked for 45s at 42°C, followed by an additional 2 min on ice. By the addition of 1 ml LB (1% W/v Bacto-Tryptone, 0.5 % w/v yeast extract and 0.5 % w/v NaCl) the cells were allowed to recover for one hour at 37°C before plating the cell suspension on LB agar plates (LB plus 1.5% w/v agar) supplemented with the appropriate antibiotic (100 µg/ml ampicillin, 50 µg/ml kanamycin, 25 µg/ml of each when double selection was required). The plates were incubated overnight at 37°C. For miniprep of plasmid DNA the resulting colonies were inoculated into 5 ml LB (plus the appropriate antibiotics) and incubated overnight at 37°C with shaking. The bacterial pellet was collected by centrifugation.

4.1.4 Isolation of plasmid DNA from *E.coli*

Plasmid DNA was recovered from bacterial pellets after transformation using QIAprep Spin Miniprep Kit (Qiagen) according to the manufacturer's instructions. The DNA was eluted from the spin column with sterile MQ water.

4.1.5 Restriction digest of plasmid DNA

Restriction digests of purified plasmid DNA were performed using commercial restriction enzymes from Fermentas and New England Biolabs according to the manufacturer's recommendation. Generally ~1 µg of plasmid DNA was digested for 1 hour at 37°C.

4.1.6 Agarose gel electrophoresis

Agarose gels were prepared with 0.6 – 2 % w/v agarose (UltraPure Agarose, Invitrogen) in 1 x TAE buffer (40 mM Tris base, 1 mM EDTA, 20 mM acetic acid, pH 8.5). A final concentration of 0.5 µg/ml ethidium bromide was added after melting the agarose and cooling to below 60°C. The DNA samples were supplemented with 6 x DNA loading buffer (10 mM Tris-HCl pH 7.6, 0.03 % bromphenol blue, 0.035 xylene cyanol FF, 60 % glycerol, 60 mM EDTA) before loading samples on the gel. As a marker the 1 kb ladder from Fermentas was used. The DNA gels were run at 100 to 120 V for 30 to 45 min in electrophoresis tanks from Amersham (HE33 or HE99X, respectively). The DNA fragments were visualized with a Multi Light Cabinet (AlphaInnotec) using AlphaEaseFC software (Aplhaimager).

4.1.7 Extraction of DNA fragments from agarose gels

DNA bands of interest were excised from the agarose gels. The DNA was recovered from the agarose gel slab using QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer's guidelines. The DNA was eluted from the spin column with sterile MQ water.

4.1.8 Ligation reaction of DNA fragments

The ligation of DNA fragments was performed using the Fermentas Rapid Ligation Kit according to the manufacturer's recommendations. The 5' ends of vector DNA were generally dephosphorylated with shrimp alkaline phosphatase (SAP, Fermentas) according to the manufacturer's instructions prior to gel extraction of the linearized DNA.

4.1.9 Sequencing of DNA

The sequencing of DNA was performed with the ABI 3730xl DNA analyzer by the sequencing facility of the Max Planck Campus, Tübingen. The

total volume of a sequencing reaction was 10 µl (0.5 µl BDT mix, 0.5 µl primer, 2 µl 5 x sequencing buffer, 2-7 µl template, sterile MQ water to 10 µl), the PCR amplification was performed at 96° C for 20 s, 50°C for 10 s and 60°C for 4 min with 30 cycles. The obtained sequences were analyzed with DNASTAR, Lasergene8 software e.g. SeqMan, MegAlign, and SeqBuilder

4.1.10 Construction of minichromosomes

The generation of the minichromosome containing a 850 bp long sequence from chromosome IV encompassing CEN4 was described earlier (Ivanov and Nasmyth, 2005; Ivanov and Nasmyth, 2007). A version without Tet operators was used to introduce BglII restriction sites using site-directed mutagenesis. A SalI digest and religation was used to remove the pUC19 sequence from the final construct prior to transformation into yeast if not stated otherwise.

4.1.11 List of minichromosomes

Table 4.1 List of minichromosomes used in this study

	Description
p1000	Sal I/Sal I *TRP1ARS1* circle with *CEN4* (850nt), the natural BglII site at *ARS1* was mutagenized.
p1001	p1000 w/ BglII site at CDEIII
p1002	p1000 w/ BglII site at CDEIII plus 50 bp downstream
p1003	p1000 w/ BglII site at CDEIII plus 100 bp downstream
p1004	p1000 w/ BglII site at CDEI
p1005	p1000 w/ BglII site at CDEI plus 50 bp upstream
p1006	p1000 w/ BglII site at CDEI plus 100 bp upstream
p1007	p1000 w/ BglII site between CDEII and CDEIII
p1008	p1001 w/ BglII site at CDEIII
p1009	p1002 w/ BglII site at CDEI plus 50 bp upstream
p1010	p1003 w/ BglII site at CDEI plus 100 bp upstream
p1031	p1007 w/ BglII site at CDEI plus 50 bp upstream
p1032	p1007 w/ BglII site at CDEIII plus 50 bp downstream
p1171	p1174 w/ BglII site at CDEIII plus 50 bp downstream

p1173	p1174 w/ BglII site between CDEII and CDEIII
p1174	Sal I/Sal I *TRP1ARS1* circle with *CEN4* (850nt); BglII site in the ARS1.

4.2 Yeast techniques

4.2.1 Budding yeast growth conditions and storage in the yeast collection

Yeast cells were grown in liquid media at 30°C with shaking at 200 rpm or on agar plates at 30°C if not stated differently. For the storage of yeast strains at -80°C the strain was grown overnight on the appropriate plate and 4 toothpicks of cells were resuspended in 1 ml of 15 % glycerol and stored at -80°C. To thaw a yeast strain a toothpick of the frozen cell suspension was streaked out on the appropriate plate and grown a 30°C if not stated differently.

4.2.2 Budding yeast media

All media was prepared using MQ water and sterilized by autoclaving if not stated differently.

4.2.2.1 Liquid media

YP:

1.1 % w/v yeast extract,

2.2 % w/v bacto-peptone,

0.0055 % w/v adenine-HCL

YPD:

YP supplemented with 2 % w/v glucose after autoclaving

YPGal:

YP supplemented with 2 % w/v galactose after autoclaving

YPRaff:

YP supplemented with 2 % w/v raffinose after autoclaving

-trp (media without tryptophan):

0.8 % w/v difco yeast nitrogen base w/o amino acids,
0.0055 % w/v tyrosine,
0.0055 % w/v adenine,
0.0055 % w/v uracil,
1.1 % w/v CAA vitamin assay (bacto casamino acids),
After autoclaving
0.01 % leucine,
2 % glucose
were added.

-ura (media without uracil):

0.8 % w/v difco yeast nitrogen base w/o amino acids,
0.0055 % w/v tyrosine,
0.0055 % w/v adenine,
1.1 % w/v CAA vitamin assay (bacto casamino acids),
After autoclaving
0.005 % leucine,
0.005 % tryptophan,
2 % glucose
were added.

-leu (media without leucine):

0.8 % w/v difco yeast nitrogen base w/o amino acids,
0.0055 % w/v tyrosine,
0.0055 % w/v adenine,
0.0055 % w/v uracil,
After autoclaving
1 % v/v -leu drop out solution (100x),
2% glucose
were added.

-leu drop out solution (100x):

0.2 % w/v arginine

0.1 % w/v histidine

0.6 % w/v iso-leucine

0.4 % w/v lysine

0.1 % w/v methionine

0.6 % w/v phenylalanine

0.5 % w/v threonine

0.4 % w/v tryptophane

-his (media without histidine):

0.8 % w/v difco yeast nitrogen base w/o amino acids,

0.0055 % w/v tyrosine,

0.0055 % w/v adenine,

0.0055 % w/v uracil,

After autoclaving

1 % v/v -his drop out solution (100x),

2% glucose

were added.

-his drop out solution (100x):

0.2 % w/v arginine

0.6 % w/v iso-leucine

0.6 % w/v leucine

0.4 % w/v lysine

0.1 % w/v methionine

0.6 % w/v phenylalanine

0.5 % w/v threonine

0.4 % w/v tryptophane

4.2.2.2 Solid media

YPD, YPGal, YPRaff, -trp, -ura, -leu, –his: same as liquid media supplemented with 2.2 % agar before autoclaving.

YPD-NAT:

YPD supplemented with 0.1 mg/l clonNAT (nourseothricin),

YPD-KAN:

YPD supplemented with 0.2 mg/l G148 (kanamycin)

YPD-HPH:

YPD supplemented with 0.3 mg/l hygromycin

spo (sporulation plates):

0.25 % w/v yeast extract,

1.5 % w/v potassium acetate,

0.1 % glucose,

2.2 % w/v agar

min (minimal plates):

0.8 % difco yeast nitrogen base w/o amino acids,

2.2 % w/v agar

4.2.3 Isolation of genomic DNA from budding yeast

Two toothpicks of yeast from an agar plate were resuspended in 180 µl SCE (1 M sorbitol, 0.1 M sodium citrate, 60 mM EDTA, pH 7), 20 µl zymolyase T100 (10 mg/ml in 20 % glucose.) and 1,5 µl β-mercaptoethanol. After incubating the suspension at 37°C for 1 hour in an Eppendorf Thermomixer Compact at 900 rpm 200 µl of lysis buffer (0.1 M Tris pH 9.0, 2 % SDS, 0.05 M EDTA) were added, vortexed and incubated for 5 min at 65°C. The suspension was allowed to cool down before adding 200 µl of potassium acetate and vortexing. The sample was centrifuged at maximal speed for 10 min at 4°C in Eppendorf 5415R centrifuge and 350 µl of the supernatant were transferred into a fresh tube with 800 µl of 100% ethanol, mixed, and centrifuged at max speed for 10 min at 4°C. The supernatant was discarded, the pellet was dried at 65°C for 10 min, and resuspended in 400 µl of TE buffer (10 mM Tris HCl [pH

7.4], 1 mM EDTA pH 8.0). For a PCR reaction 1 µl of isolated genomic DNA were used in 25 µl total.

4.2.4 Transformation of budding yeast

The yeast cells were grown in 5 ml of YPD overnight. The next day 50 ml of YPD were inoculated at OD_{600} 0.2 and harvested at OD_{600} 0.8 by centrifuging at 3000 rpm for 3 min in an Eppendorf 5810R centrifuge. The cells were washed in 1 ml of lithium acetate twice, centrifuged at RT for 1 min at 1000 g in a Eppendorf 5415R and resuspendend in a 1:1 volume of lithium acetate (for a 50 ml culture usually 90 µl). 8 µl of salmon sperm DNA, 90µl 50 % PEG 3350, 24 µl of the cell suspension were mixed, and 8 µl of DNA (~200 ng of linear or plasmid DNA) added, shortly vortexed, and incubated at RT. After 30 min 12 µl of 60% glycerol were added and incubated for half an hour before a heat shock for 10 min at 42°C. For selective media containing antibiotics (e.g. KAN, NAT, HPH) the transformed cells were incubated in YPD for 3 h at 30°C and plated. For selective media without antibiotics the cells were plated immediately. The plates were incubated for 2-3 days at 30°C. The transformants were checked for the correct integration or the presence of episomal plasmid by PCR and plasmid stability assay. Expression of tagged proteins was tested by Western blot.

4.2.5 Stability assay for strains transformed with a minichromosome

To test for the correct transformation of a strain with the minichromosomes, the individual colonies were plated on YPD and grown overnight. The next day, the cells were singled on YPD and incubated overnight before replica plating the singled cells on –trp plates. After another overnight incubation the singled cells of YPD plates and the –trp replica plate were compared to each other. The transformation of a strain with the minichromosome was considered successful, when some colonies were unable to grow on the –trp plate after the non-selective growth on YPD plates. The minichromosomes are unstable and yeast strains carrying them were subsequently grown in the selective media.

4.2.6 Crossing of budding yeast strains

Small amounts of two haploid yeast strains of opposite mating types (MAT a and MAT alpha) were each resuspended in 50 µl of 1 M sorbitol and 20 µl aliquots of each suspension were carefully combined and mixed together in an Eppendorf tube. 10 µl were carefully pipetted on a pre-warmed YPD plate. For isolating the zygotes, a micromanipulator MSM System 300 TSA microscope (Singer Instruments) was used. The plate was incubated for 5 h and a small portion of cells was carefully resuspended in 90 µl of 1 M Sorbitol to dilute the cells and applied on a fresh pre-warmed YPD plate from which the zygotes were picked up with the micromanipulator. For the selection of the diploid yeast cells on selective media the YPD plate with the mixed suspension of the parental strains was incubated overnight and the yeast cells were singled on the appropriate selective plate and replica plated onto the second selective plate the next day.

4.2.7 Tetrad dissection of budding yeast strains

Diploid yeast cells were grown on sporulation plates for 2-3 days. A small amount of cells was carefully resuspended in 90 µl of 1 M sorbitol supplemented with 10 µl of zymolyase T100 (10 mg/ml in 20 % glucose) and incubated for 20-30 minutes at 30°C. 10 µl of the reaction mix were applied on an YPD plate. The tetrads were dissected using a MSM System 300 TSA microscope (Singer Instruments). The dissection plates were incubated at 25-30°C for 2-3 days and analyzed by streaking colonies on an YPD plate and replica plating it to the appropriate selective plates. For the mating type identification the YPD plate was replica plated onto an YPD plate with a suspension of the strain 216 or 217, incubated for at least 5 hours before replica plating on minimal plates. The plates were analyzed and the desired cloned was added to the yeast strain collection.

4.2.8 Budding yeast strain construction

All strains are isogenic in the W303 background and unless indicated otherwise have the genotype *MATa ade2-1 trp1-1 can1-100 leu2-3,112, his3-11,15 ura3* GAL psi^{+}. The strains are described in Table 4.2.

4.2.8.1 Epitope tagging of CENP-A^{Cse4}

The *CSE4* gene with flanking sequences (450 bp upstream and 300 bp downstream of the coding sequence) was cloned into the integrative plasmid YIplac128 (Gietz and Sugino, 1988). Either a 225 bp NheI fragment encoding six copies of the hemagglutinin epitope (HA), a 228 bp or a 121 bp XbaI fragment encoding six or three copies of the Myc epitope, respectively, was inserted in the natural internal XbaI site within the *CSE4* open reading frame (Meluh et al., 1998). The resulting plasmid was integrated into the *LEU2* locus on chromosome III. The endogenous untagged *CSE4* gene was replaced by the *NAT* marker cassette of plasmid pAG32 according to (Goldstein and McCusker, 1999). The successful tagging was confirmed by PCR, sequencing and Western blot analysis.

For the Cse4 constructs with the substitution of alanine 111 to cysteine (A111C) the integrative vector either containing Cse4-Myc6 or Cse4-Myc3 was subjected to a site-directed mutagenesis and the resulting product was integrated in the appropriate yeast strain.

4.2.8.2 Epitope tagging of the core histones and the protein Scm3

All other histones, besides Cse4, were tagged at the C-terminus with HA3 using the plasmid pYM2-HIS3MX6 according to (Knop et al., 1999) and the second gene was either left untagged (H4) or deleted (H2A, H2B, H3) using the antibiotic resistance cassettes amplified from plasmids pAG25 (H2A and H2B) and pAG32 (H3) according to (Goldstein and McCusker, 1999). The H3-FLAG strain was created with a modified protocol of (Knop et al., 1999). The FLAG epitope was included in the sequence of one of the primers used to amplify the *HIS3* marker cassette. The Primers included overhangs complimentary to the sequences at the 3'end of the H3 gene and its 3' UTR to target the integration at the H3 locus. Scm3 was tagged with HA6 using a cassette from the plasmid pYM3-HIS3MX6 according to (Janke et al., 2004). The successful tagging was confirmed by PCR, sequencing and Western blot analysis.

4.2.8.3 Construction of the strain with BglII sites flanking CEN4 on the native chromosome IV

To introduce BglII restriction sites flanking the centromeric DNA on the native chromosome IV, the region of CEN4 +/- 200bp was cloned into the PvuII site of pOM10 (courtesy of Anne Spang) (Gauss et al., 2005) and BglII sites were introduced by mutagenesis. A yeast strain was transformed with a PCR product containing the CEN4 DNA with BglII sites, a CEN flanking sequence, and the KANMX6 marker. The BglII flanked CEN4 DNA was recombined into the endogenous locus and the marker cassette was removed according to (Gueldener et al., 2002) by transforming the resulting strain with the plasmid pSH47 containing the Cre recombinase gene. Upon expression of Cre recombinase the marker cassette was looped out leaving 85 bp of the pOM10/loxP sequence 200 bp downstream of CDEIII. Clones, which lost pSH47 after growth in non-selective media and were unable to grow on KAN-containing plates were chosen. The complete CEN4 region was sequenced.

4.2.8.4 List of strains

All strains are isogenic in the W303 background and unless indicated otherwise have the genotype *MAT a ade2-1 trp1-1 can1-100 leu2-3,112, his3-11,15 ura3* GAL psi^{+}.

Table 4.2 List of yeast strains used in this study

	Genotype
216	*MAT a, his1,* Nasmyth lab, DC14a background
217	*MAT alpha, his1,* Nasmyth lab, DC14a background
1016	*Mat a/Mat alpha diploid, ade2-1/ade2-1, trp1-1/trp1-1, can1-100/can1-100, leu2-3,112/leu2-3,112, his3-11,15/his3-11,15, ura3-52/ura3-52* (wildtype)
1021	wild type
1266	*HHT1-HA3::HIS3*
1268	*hht2::HPH HHT1(A111C)-HA3::KAN::HIS3*
1407	*hht2::HPH HHT1-HA3::HIS3*
1498	*cse4::NAT CSE4-HA6::LEU2*

1576	*hta2::NAT HTA1-HA3::HIS3*
1577	*HHF1-HA3::HIS3*
1587	*htb2::NAT HTB1-HA3::HIS TetR-GFP-TAP::LEU2*
1593	*SCM3-HA6::HIS3*
1837	*HHT1-FLAG1::HIS3*
1923	*MAT alpha cse4::NAT CSE4-Myc6::LEU2*
1924	*cse4::NAT CSE4(L204C)-Myc3::LEU2*
1949	*cse4::NAT CSE4(L204C)-Myc3::LEU2 hht2::HPH HHT1(A111C)::KAN*
1953	*cse4::NAT CSE4(L204C)-Myc3::LEU2 hht2::HPH HHT1(A111C)-HA3::KAN::HIS3*
1955	*cse4::NAT CSE4(L204C)-Myc6::LEU2 hht2::HPH HHT1(A111C)-HA3::KAN::HIS3*
2042	*hht2::HPH HHT1-HA3::HIS3 CEN4* flanked with BglII +/-50 bp
2043	*cse4::NAT CSE4-HA6::LEU2 CEN4* flanked with BglII +/-50 bp
2059	*MAT alpha CEN4* flanked with BglII +/-50 bp
2070	*HTZ1-HA3::HIS3*
2300	*cse4::NAT CSE4-Myc6::LEU2 hht2::HPH HHT1-HA3::HIS3*
2561	*cse4::NAT CSE4-Myc6::LEU2 hht2::HPH HHT1-HA3::HIS3 CEN4* flanked with BglII +/-50 bp
2562	*cse4::NAT CSE4-Myc6::LEU2 CEN4* flanked with BglII +/-50 bp

4.2.9 Cell cycle arrest of budding yeast

4.2.9.1 Arrest in G1 with alpha factor

YPD media was inoculated with the pre-culture at an OD_{600} of 0.05 and grown until an OD_{600} 0.2. The culture was supplemented with 2 µg/ml alpha factor and grown for 1 hour. After 1 hour, additional 1.5 µg/ml alpha factor were added followed by an additional hour of incubation.

4.2.9.2 Arrest in G2 with nocodazole

For a G2 arrest of the cell cycle, 15 µg/ml nocodazole and 10 µg/ml benomyl were added to a yeast culture at an OD_{600} of 0.65 in YPD medium, and cells were incubated for 2 hours.

4.2.10 FACS analysis of an arrested yeast cell culture

To monitor the cell cycle stage of a yeast cell culture an aliquot of 1 ml was collected and spun down for 1 min at 1000 g. The pellet was resuspended in ice cold 70 % ethanol and stored at -20°C. To digest the RNA the cells were resuspended in 1 ml of RNase A buffer (50 mM Tris [pH 7.5], 0.2 mg/ml RNase A Fermentas) and incubated overnight at 37°C. The cells were collected and resuspendend in propidium iodide buffer (200 mM Tris [pH7.5], 211 mM NaCl, 78 mM $MgCl_2$, 27 µg/µl propidium iodide) and sonicated (Sonifier S-450 analogue, Branson Danbury USA) for ~5 seconds, output control 2-2.5. The sonicated cells were checked by microscopy and the cell suspension was diluted 1:20 with sheath fluid (Partec). At least 10,000 cells were counted with the CyFlow SL machine and the FloMax software (Partec). The FACS graphs were assembled with the WIN MDI Software.

4.2.11 Spheroplasting of budding yeast cells

To prepare spheroplasts of yeast cells the cell wall was digested with lyticase (Sigma, L2524). A modified protocol of (Deshaies and Kirschner, 1995) was used. Generally, for a culture of 50 ml (OD_{600} 1.6), the cells were harvested, washed twice in cold water, resuspendend 45 ml in pre-spheroplasting buffer (0.1 M Tris [pH 9.4], 10 mM DTT), and incubated for 15 min at room temperature. Subsequently, the cells were washed with cold water, resuspended in 35 ml prewarmed spheroplasting buffer (1 M sorbitol, 50 mM Tris [pH7.5], 1 mM CaCl2, 1 mM $MgCl_2$, 57 U/ml lyticase), incubated at 30°C in a water bath with shaking for 30 min, and the tubes were inverted every 10 min to keep the cells in suspension. The spheroplasting was checked under the microscope, cells should look round and lyse easily by the addition of 1 % sarcosyl. The spheroplasts were harvested at 4300 g for 6 min and washed in cold 1 M sorbitol before continuing with an experiment.

A yeast culture of 50 ml (OD_{600} 1.6) can also be spheroplasted in 2 ml Eppendorf tubes by resuspending the cell pellet in 1.5 ml pre-spheroplasting buffer and 1.5 ml spheroplasting buffer supplemented with 600 U/ml lysticase. For larger yeast cultures of 150 to 300 ml (OD_{600} 1.6) the spheroplasting was

carried out with in 35 ml spheroplasting buffer supplemented with 400 U/ml lyticase.

4.3 Biochemical techniques

4.3.1 Covalent coupling of antibody to protein A Dynabeads

The protein A Dynabeads (Invitrogen) were washed with PBS containing 0.02 % Tween 20 and 0.1 mg/ml BSA (1x BSA NEB). The beads (1 ml of the original suspension) were resuspended in PBS with 0.02 % Tween 20 and 240 µg of antibody and either incubated for 45 min at room temperature or overnight at 4°C. The beads were washed once in PBS with 0.02 % Tween 20 and two times in 0.2 M triethanolamine [pH 8.2]. For the cross-linking of the antibody to Protein A the beads were incubated for 30 min to 1 hour at room temperature in 20 mM of DMP (dimethyl pimelimidate) in 0.2 M triethanolamine [pH 8.2] (5.18 mg of DMP in 1 ml). The cross-linking reaction was stopped by washing the beads with 50 mM Tris [pH 7.5] and incubation of the beads for 15 min with 50 mM Tris [pH 7.5]. To remove antibody that was not covalently coupled, the beads were washed once with 0.1 M citric acid for 2 min and stored afterwards in PBS with 0.02 % Tween 20 and 0.1 mg/ml BSA.

4.3.2 Chromatin immunoprecipitation experiments

4.3.2.1 ChIP without cross-linking chromatin

Yeast strains transformed with the minichromosome were grown overnight in synthetic medium without tryptophan at 30°C, were inoculated into 150 ml of fresh medium to a final OD_{600} of 0.2, and grown until the OD_{600} reached 1.6. Yeast strains without minichromosomes were grown in YPD. The prepared spheroplasts were lysed for 30 min on ice in 2.5 ml of lysis buffer (25 mM HEPES/KOH [pH 8.0], 50 mM KCl, 10 mM $MgSO_4$, 10 mM Na citrate, 25 mM Na sulfite, 0.25 % TritonX-100, 1 mM PMSF, 3 mM DTT, 1x complete EDTA-free protease inhibitors (Roche) and 100 µg/ml RNase A). The lysate was cleared by centrifugation at 10,000 rpm for 5 min in an Eppendorf 5415R microcentrifuge. For the DNA digest, the lysate was incubated with 1 unit/µl of BglII (NEB) for 2 hours with rotation at 4°C before adding NaCl to a final concentration of 300

mM to stop the digest. For strains with BglII sites on chromosome IV the crude lysate was incubated with BglII and cleared after 2 hours of digestion. Pre-cleared lysate (2ml) was incubated with 25 µg of anti-HA (12CA5) antibody and 0.5 ml suspension of protein A Dynabeads (Invitrogen) overnight. For the ChIP experiment with FLAG tagged histone H3, the yeast cell lysate was incubated with 25 µg of FLAG M2 antibody (Sigma) and protein G Dynabeads (Invitrogen). Beads were washed 3 times with 1.5 ml of the lysis buffer with 300 mM NaCl. Isolated DNA was eluted off the beads two times with 250 µl of 50 mM Tris [pH 8.0], 10 mM EDTA and 1 % SDS at 65°C. The samples were adjusted to a final concentration of 1 % SDS, extracted twice with phenol/chloroform/isoamyl alcohol (25:24:1), ethanol precipitated in the presence of 20 µg glycogen (Roche), and samples were dissolved in 20-40 µl TE.

4.3.2.2 ChIP with cross-linking chromatin

For ChIP experiments with cross-linking, the DNA was digested in the lysate with BglII for 5 min at 37°C, the digest was stopped by adding 300 mM NaCl, and the chromatin was immediately cross-linked by adding 0.1 % formaldehyde for 30 min and subsequently 125 mM glycine for 15 min on ice. The cross-linked lysate was incubated with protein A Dynabeads covalently coupled to either anti-HA (12CA5, Roche) or anti-Myc (9E11, Santa Cruz) antibody. For the sequential immunoprecipitation experiment the chromatin was eluted off the beads as described above, diluted to 0.1 % SDS with lysis buffer supplemented with 300 mM NaCl, and immunoprecipitated with protein A Dynabeads covalently coupled to anti-HA antibodies (12CA5). The DNA was eluted off the beads as above. The samples were adjusted to a final concentration of 1 % SDS, extracted twice with phenol/chloroform/isoamyl alcohol (25:24:1), ethanol precipitated in the presence of 20 µg glycogen (Roche), and samples were dissolved in 20-40 µl TE.

4.3.3 Sample preparation for quantitative PCR (qPCR)

For qPCR experiments the isolated DNA was size fractionated on a 2 % agarose gel (Certified Low Range Ultra Agarose, Bio-Rad) with a DNA ruler (Low Molecular Weight DNA Ladder, NEB) and excised from the agarose gel to separate from uncut and linear minichromosomes. The gel extraction was

performed with the QIAquick Gel Extraction Kit (Qiagen) for the 214 bp CEN4 DNA fragment and the QIAEX II Gel Extraction Kit (Qiagen) was used to extract the 139 bp CDEI/II and the 77 bp CDEIII fragments according to the manufacturer's guidelines. The DNA was eluted with PCR grade water and subjected to the qPCR experiment.

4.3.4 LightCycler qPCR

The quantitative PCR was performed with the LightCycler® 480 SYBR Green I Master solutions with the LightCycler® 480 Instrument II, 96-well block (Roche) and the data was analyzed using the LightCycler® 480 Instrument II software (software release 1.5.0). The master mix was prepared according to the manufacturer's instructions. A total volume of 20 µl per sample contained 3 µl PCR grade water, 2 µl of primers (10 µM, 10 fold concentrated) 10 µl of SYBR Green I Master and 5 µl of the DNA template. The standard program was used with 45 amplification cycles unless otherwise stated. Briefly, pre-incubation for 95°C for 5 min, amplification for 45 cycles with 95°C for 10 s, 60°C for 30 s, and 72°C for 20 s, melting curve with 95°C for 5 s, 65°C for 1 min, and cooling to 40°C)

For the 214 bp CEN4 DNA the primers AGTAACTTTTGCCTAAATCAC (sense) and TAGGTAGTGCTTTTTTTCCA (anti-sense) were used. The 139 bp CDEI/II fragment was amplified with the primers TAGTAACTTTTGCCTAAATC (sense) and TAATAAATAAATTATTTCATTTATGTTT (anti- sense) in 55 cycles and the 77 bp fragment of CDEIII was amplified with the primers TGTTTATGATTACCGAAACA (sense) and TTAGGTAGTGCTTTTTTTCC (anti-sense). The resulting qPCR products were checked by sequencing.

4.3.5 Southern blot analysis

4.3.5.1 Capillary Southern blot transfer and radioactive detection

For the analysis of the uncut, linear and large fragments of the minichromosomes the samples were separated on a 0.8 % or 1 % agarose gel with 0.5 µg/ml ethidium bromide at 120 V for 2.5 h and a capillary transfer was carried out to either Hybond-N+ (GE) under neutral conditions or to Hybond-XL (GE) under alkaline conditions according to the manufacturer's guidelines.

After the Southern transfer of the DNA from the agarose gel to the membrane, the blot was cross-linked in a UV Stratalinker 2400 (Auto Cross Link setting) and rinsed with 2 x SSC (20 x SSC 88.23 g/l tri-sodium citrate, 175.32 g sodium chloride [pH 7-8]). The blot was hybridized with a specific radioactive probe for the *TRP1* gene or the CEN4 sequence at 65°C in hybridization buffer (per blot 7 ml of Dextran buffer (200 g/l Dextran, 2 % SLS, 12 x SSC), 6 ml of MQ water, 60 µl salmon sperm DNA, Invitrogen) overnight. The length of the DNA fragments that were used as templates to synthesize the hybridization probe were 560 bp for CEN4 and 760 bp for *TRP1*. The probes were labeled with [α-^{32}P]dCTP (Hartmann Analytic) using the Prime-It II Random Primer Labeling Kit from Stratagene according to the manufacturer's guidelines and purified from unincorporated [α-^{32}P]dCTP using a Nick Column G50 Sepharose DNA grade (GE). After the hybridization, the blot was washed three times for 30 min at 65°C in 2 x SSC, 1 % SDS buffer. The blots were exposed to storage phosphor screen (Molecular Dynamics) overnight, scanned on a Personal Molecular Imager (BIORAD), and the bands were quantified with QuantityOne 4.6.7.

4.3.5.2 Electrophoretic Southern blot transfer and non-radioactive detection

For the detection of the small CEN4 DNA fragments or the CDEI/II fragments a double-DIG labeled CDEI/II locked nucleic acid (LNA) probe (AAAGTTGATTATAAGCATGTGAC, Exiqon) was employed. The DNA samples were supplemented with 6x DNA loading dye, boiled and rapidly cooled down on ice before separating on a denaturing 6 % TBE polyacrylamide gel at 100 V for 1 h in 1x TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA, [pH 8.3]). The polyacrylamide gel was incubated for 10 min in 1x TBE containing 0.2 mg/ml ethidium bromide to visualize nucleic acids and subsequently electrophoretically transferred in 1x TBE buffer at 4°C to Hybond-N+ membrane at 80 V for 1 hour in 1x TBE in the Trans-Blot System (Biorad). After the Southern transfer the membrane was rinsed in 2x SSC and UV cross-linked. The hybridization was performed with 14 ng/ml of the double DIG labeled LNA probe using the reagents of the DIG High Prime DNA Labeling and Detection Starter Kit II (Roche) at 55°C overnight according to the instructions. The

hybridized Southern blot was incubated with Anti-Digoxygenin-AP, Fab fragments (Roche), detected using CSPD (Roche) according to the manufacturer's recommendations and exposed to Hyperfilm ECL (Amersham), which was developed with a developing machine (Konica Minolta, SRX-101A). Depending on the signal strength exposure times between 30 min and 24 h were necessary.

4.3.6 Ex vivo cross-linking of histones on chromatin

A yeast culture of 50 ml YPD was harvested at OD_{600} 1.6. Spheroplasting was carried out using the same procedure as for ChIP. Spheroplasts were washed in 1 M sorbitol and lysed in 1 ml cold reaction buffer (25 mM sodium phosphate [pH 7.0], 100 mM KCl, 2.5 mM $MgCl_2$, 0.25 % TritonX-100) for 15 min on ice. The chromatin was pelleted using a low-speed centrifugation (1500g, 1 min) and the supernatant was discarded. The chromatin pellet was then resuspended and aliquoted (4 times 250 µl) in the reaction buffer with varying concentrations of the cross-linker. DTNB (5,5'-dithiobis-(2-nitrobenzoic acid), Sigma) was prepared as a 50 mM stock in DMSO and diluted into the reaction mixture as appropriate (0 mM (DMSO alone), 0.05 mM, 0.5 mM and 5 mM final DTNB). Cross-linking was allowed to proceed for 1 hour on ice. The chromatin was then pelleted by centrifugation and resuspended in SDS-PAGE loading dye (4x SDS loading dye: 250 mM Tris, 40 % glycerol, 4 % SDS, 2 % bromphenol blue) without DTT or β-mercaptoethanol. The samples were subjected to SDS-PAGE and Western blot analysis.

4.3.7 Preparation of protein extracts from budding yeast using trichloroacetic acid (TCA) precipitation

Cell extracts were prepared according to (Reid and Schatz, 1982). Briefly, 20 ml of a liquid culture of OD_{600} 0.6 were harvested and the cells were washed in 1 ml of trichloroacetic acid (10 % TCA, ice cold). The cells were resuspended in 200 µl of 10 % TCA and the suspension transferred to a Vetter tube (provided by Roland Vetter Laborbedarf OHG, Ammerbuch) filled with 1.2 ml of acid washed glass beads (Sigma-Aldrich). The cells were disrupted by beads beating at 4 °C in a FastPrep™ machine (Qbiogen, MPBiomedicals, Heidelberg) for 40 s at speed 6.5. The tube was punctured at the bottom and placed in an empty 1.5

ml Eppendorf-tube for centrifugation (3 min, 1200 rpm) to harvest the lysed yeast cell extract. The glass beads were washed with an additional 200 µl of 10 % TCA and centrifuged. The collected cell lysate was centrifuged at maximal speed for 10 min at room temperature, the supernatant was discarded and the pellet containing the precipitated protein was vigorously resuspended in 100 µl of 2 x SDS loading dye, which turned from blue to yellow. The acidic pH was neutralized by the addition of 25 µl of 2 M Tris base (the loading dye turned back to blue). The samples were vortexed briefly, boiled for 10 min, centrifuged (10 min, maximal speed, room temperature), and the protein-containing supernatant was transferred to a new tube. Prior to SDS-PAGE and Western blot the sample was boiled and spun again.

4.3.8 Western blot analysis

For the analysis of proteins by Western blot the protein samples were boiled for 5 min with SDS loading dye (4x SDS loading dye: 250 mM Tris, 40 % glycerol, 4 % SDS, 2 % bromphenol blue, w/ or w/o 350 mM β-mercaptoethanol) and separated on a SDS-PAGE gel with the appropriate polyacrylamide percentage at 110 V for 90 min in 1x SDS running buffer (118 mM Tris-base, 40 mM glycine, 0.1 % SDS). The Precision Plus Protein Standard (BioRad) was used as a molecular weight marker. The SDS-PAGE gel was incubated in 1x transfer buffer (48 mM Tris-base, 39 mM glycine, 0.0375 % SDS, 20 % methanol) for 15 min, the Western blot transfer was performed with the methanol-activated PVDF membrane (0.45 µm, BioRad), and transfer buffer-soaked blotting paper in a SemiDry Transfer Cell (BioRad) for 15 min at 15 V or in an ECL SemiDry Transfer Unit (Amersham Bioscience) for 1 h at 30 mA. The PVDF membrane was blocked for 30 min in PBST (0.05% Tween 20 in phosphate buffers saline) with 0.5 % skim milk and probed for 1h with primary and secondary (anti-primary HRP conjugated) antibody at the appropriate concentration in PBST with 0.05 % skim milk. After each antibody incubation the blot was washed three times with PBST for 15 min. The Western blot was analyzed with ECL Western detection system according to the manufacturer's recommendations (Amersham), exposed to Hyperfilm ECL (Amersham) or

BioMax MR Film, High Resolution (Kodak), and developed with a developing machine (Konica Minolta, SRX-101A).

Primary and secondary antibodies used for Western blot were:

Anti-	Host	Dilution
FLAG (M2, Sigma)	mouse	1:1.000 – 1:5.000
HA (16B12, Covance)	mouse	1:1.000 – 1:10.000
HA (4A6, Millipore)	mouse	1:500 – 1:2.000
HA (C29F4, CST)	rabbit	1:1.000 – 1:20.000
HA (ICL, Inc.)	chicken, HRP-conjugated	1:2.000 – 1:5.000
HIS-tag (Penta-His, QIAGEN)	mouse	1:1000 – 1:20.000
Myc (71D10, CST)	rabbit	1:1.000 – 1:10.000
mouse (ECL antibody, GE)	sheep, HRP-conjugated	1:5.000
rabbit (ECL antibody, GE)	goat, HRP-conjugated	1:5.000

4.4 Recombinant protein expression

4.4.1 List of plasmids for the expression of recombinant proteins

The DNA sequences of the budding yeast genes *CSE4*, *HHT1* (H3), and *HHF1* (H4) were codon optimized for expression in *E.coli* and synthesized by GenScript. Different constructs were created by cloning the codon-optimized sequence into the desired expression vector (either pETDuet-1 or pRSF-Duet-1, Novagen) after DNA amplification with primers that contained overhangs with the appropriate restriction sites. Prior to cloning into the final vector the PCR products were sub-cloned into pJET1.2 using the CloneJET PCR Cloning Kit (Fermentas) according to the manufacturer's instructions to simplify the restriction digest. All constructs were sequenced.

Table 4.3 List of plasmids with constructs for recombinant expression of proteins used in this study

	Description
1361	Histone H4 starting with 1HA-tag and lysine 20 (HA-K20-HHF1) cloned into NcoI and NotI site of pETDuet-1 (Amp)
1363	Histone H3 with an N-terminal biotinylatable AviTag and alanine

	111 mutated to cysteine (AviTag-Tev-HHT1 A111C) cloned into NcoI and NotI site of pRSF-Duet-1 (KAN)
1367	CENP-A^{Cse4} with an N-terminal 6xHis tag and leucine 204 mutated to a cysteine (6xHIS-CSE4 L204C) cloned into NdeI and XhoI site of pRSF-Duet-1 (KAN)
1368	CENP-A^{Cse4} starting with leucine 81 (6xHIS-L81-CSE4 L204C) cloned into NdeI and XhoI site of pRSF-Duet-1 (KAN)
1369	CENP-A^{Cse4} starting with tyrosine 132 (6xHIS-Y132-CSE4 L204C) cloned into NdeI and XhoI site of pRSF-Duet-1 (KAN)
1487	CENP-A^{Cse4} starting with aspartate 150 (6xHIS-D150-CSE4 L204C) cloned into NdeI and XhoI site of p1363 (KAN)
1488	CENP-A^{Cse4} starting with aspartate 150 (6xHIS-D150-CSE4 L204C) cloned into NdeI and XhoI site of pETDuet-1 (AMP)

4.4.2 Protein expression in *E.coli*

For the overexpression of the desired construct the appropriate plasmid was transformed into the *E.coli* strain BL21 (DE3). For the expression of biotinylated histone H3 the bacteria were also transformed with the commercially available plasmid pBirACm (Avidity, L.L.C) to overexpress the biotin ligase BirA. The induction procedure for AviTagged proteins was carried out according to the manufacturer's protocols (Avidity, L.L.C.). Briefly, an overnight culture was grown in TYH media (20 g/l tryptone, 10 g/l yeast extract, 11 g/l HEPES, 5 g/l NaCl, 1 g/l $MgSO_4$, [pH 7.2 – 7.4 with KOH]) supplemented with the appropriate antibiotic (10 µg/µl chloramphenicol, 25 µg/µl kanamycin, and/or 25 µg/µl ampicillin) at 37°C at 220 rpm. The overnight culture was diluted 1:200 into TYH (without antibiotic) supplemented with 0.5 % glucose and grown at 37°C at 220 rpm. At OD_{600} 0.7 50 µM of d-biotin and 1.5 mM of IPTG were added and the protein expression was induced overnight. Samples were taken prior induction and 3h, 5h, and overnight after induction. Samples of the bacterial culture were collected by centrifugation and resuspended in SDS-PAGE loading buffer with and without β-mercaptoethanol. The next day the cells were harvested at 5000 g for 30 min, resuspended in lysis buffer (20 mM HEPES, 300mM NaCl, 5 mM imidazole, 5 % glycerol, and 1x

Complete Protease Inhibitor Cocktail, Roche) and lysed using EmulsiFlex-C3 high pressure homogenizer with a pressure of ~12.000 psi. The bacterial extracts were centrifuged two times at 30.000 g to clear the lysate. Samples were taken for the pellet and the supernatant, samples were separated on a 15 % SDS-PAGE, and either analyzed with Coomassie Blue staining (Roti-Blue, Roth) according to the manufacturer's guidelines or by Western blot analysis. Western blots were analyzed with Streptavidin-HRP (Pierce) for Avitag-H3 constructs, with anti-Penta-His antibody (Qiagen) for 6His-Cse4 constructs, and with anti-HA for HA-H4 constructs.

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6 Appendix

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Histone H3 Localizes to the Centromeric DNA in Budding Yeast

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Abstract

During cell division, segregation of sister chromatids to daughter cells is achieved by the poleward pulling force of microtubules, which attach to the chromatids by means of a multiprotein complex, the kinetochore. Kinetochores assemble at the centromeric DNA organized by specialized centromeric nucleosomes. In contrast to other eukaryotes, which typically have large repetitive centromeric regions, budding yeast CEN DNA is defined by a 125 bp sequence and assembles a single centromeric nucleosome. In budding yeast, as well as in other eukaryotes, the Cse4 histone variant (known in vertebrates as CENP-A) is believed to substitute for histone H3 at the centromeric nucleosome. However, the exact composition of the CEN nucleosome remains a subject of debate. We report the use of a novel ChIP approach to reveal the composition of the centromeric nucleosome and its localization on CEN DNA in budding yeast. Surprisingly, we observed a strong interaction of H3, as well as Cse4, H4, H2A, and H2B, but not histone chaperone Scm3 (HJURP in human) with the centromeric DNA. H3 localizes to centromeric DNA at all stages of the cell cycle. Using a sequential ChIP approach, we could demonstrate the co-occupancy of H3 and Cse4 at the CEN DNA. Our results favor a H3-Cse4 heterotypic octamer at the budding yeast centromere. Whether or not our model is correct, any future model will have to account for the stable association of histone H3 with the centromeric DNA.

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Introduction

During eukaryotic cell division sister chromatids, containing identical copies of genetic information, are pulled apart and driven towards opposite spindle poles by the microtubules of the mitotic spindle, which attach to the centromeric DNA sequences of the sisters via kinetochore protein complexes. It is imperative for proper chromosomal segregation that each chromosome assembles the kinetochore only at one site. The sites of kinetochore assembly are marked by specialized nucleosomes. Budding yeast represents the simplest case in which a single microtubule attaches to the so-called "point" kinetochore assembled around a single centromeric nucleosome. More complicated "regional" centromeres of most other eukaryotes are composed of arrays of specialized centromeric nucleosomes interspersed with conventional nucleosomes [1] and support the assembly of several microtubule attachment sites.

Centromeric nucleosomes were reported to have histone H3 substituted by a histone variant, CENP-A, called Cse4 in budding yeast [2]. It displays more than 60% similarity with the conventional histone H3 within the histone fold domain and has an additional N-terminal extension [3]. CENP-A has been demonstrated to co-purify with a subset of kinetochore proteins and is likely to provide interaction surfaces for kinetochore assembly [4,5]. Recruitment of CENP-A to centromeric DNA requires the CENP-A targeting domain (CATD), comprised of loop1 and the α2-helix [6,7], and is regulated by a number of other proteins [8]. One example is the non histone protein Scm3 (HJURP in human [9]), which is believed to be a histone chaperone required for recruitment of CENP-A to centromeres [10 18]. CENP-A overexpression in metazoans [19] and budding yeast [20] leads to its mislocalization. In budding yeast mislocalized Cse4 is very unstable [21]. Although budding yeast [22] and fission yeast [14,23,24] appear to be an exception, in several organisms CENP-A is loaded on the DNA outside of S phase, in anaphase of mitosis or the following G1 [25,26], when it is proposed to replace histone H3.

Despite a significant progress in the field, the exact function of CENP-A at the centromere remains a mystery. CENP-A and H4 were reported to form a more compact and conformationally more rigid heterotetramer compared to the heterotetramer of histones H3 and H4 [6,27]. However, the significance of the structural differences between H3 and CENP-A to their function is unknown. Even the question of the exact composition and localization of centromeric nucleosomes has not been resolved to date and remains the subject of controversy [28]. Besides an octamer composed of two molecules each of CENP-A, H2A, H2B and H4, a hexamer model in which Scm3 replaces H2A and H2B [11,17] and a hemisome model which proposes a tetramer consisting of one copy each of Cse4, H4, H2A and H2B [29 32] were also proposed. Regional centromeres of higher eukaryotes can accommodate different versions of CENP-A-containing nucleosomes. While budding yeast with their point centromeres is an appealing model system to study the centromeric nucleo-

Author Summary

During cell division, replicated DNA molecules are pulled to daughter cells by microtubules, which originate at the spindle poles and attach to a multiprotein complex, the kinetochore. The kinetochore assembles at a special region of the chromosome, termed the centromere. The kinetochore is comprised of more than 50 different proteins whose precise functions are far from being fully understood. The kinetochore assembles on the foundation of a specialized centromeric nucleosome. A nucleosome is a complex of eight subunits, termed histones, which compacts the DNA by wrapping it around itself in 1.7 turns of a superhelix. The centromeric nucleosome is very special, and its stoichiometry and structure are a subject of intense debate. It is believed that the centromeric nucleosome is devoid of histone H3 and instead contains its variant, termed CENP-A in vertebrates or Cse4 in budding yeast. Here we report that in budding yeast both CENP-A and histone H3 localize to a small centromeric DNA fragment that, due to its size, cannot accommodate more than a single nucleosome. Our results necessitate a revision of what is known about the structure of the inner kinetochore and the role of CENP-A in its assembly.

some, it is possible that the yeast centromeric nucleosome might also possess unique features.

Here we report the results of our analysis of the yeast centromeric nucleosome using a novel chromatin immunoprecipitation technique and discuss them in the context of the previously proposed models of the CENP-A containing nucleosome.

Results

High-resolution chromatin immunoprecipitation technique

The composition of the centromeric nucleosome was previously analyzed by means of chromatin immunoprecipitation (ChIP) [11,12] in yeast. In a conventional ChIP approach proteins are chemically cross-linked to DNA, the chromatin is fragmented by sonication to about 500 bp size, and immunoprecipitated fragments are identified in PCR or microarray hybridization assays. This approach suffers certain drawbacks when applied to the centromere. The DNA fragment size is much larger than the region accommodated by a conventional nucleosome (146 bp), which limits the resolution. This problem can in principle be overcome by the treatment of chromatin with micrococcal nuclease, which specifically digests the internucleosomal linker DNA. However the size of kinetochore footprint is highly variable depending on the digest conditions [33,34] and apparently poses an accessibility problem for antibodies since the efficiency of the co-immunoprecipitation of the CEN DNA with canonical histones is very low compared to pericentric regions [11,12,35]. In addition, PCR with a specific pair of primers or microarray hybridization detect larger DNA fragments without identifying them by size, which imposes further limits on resolution.

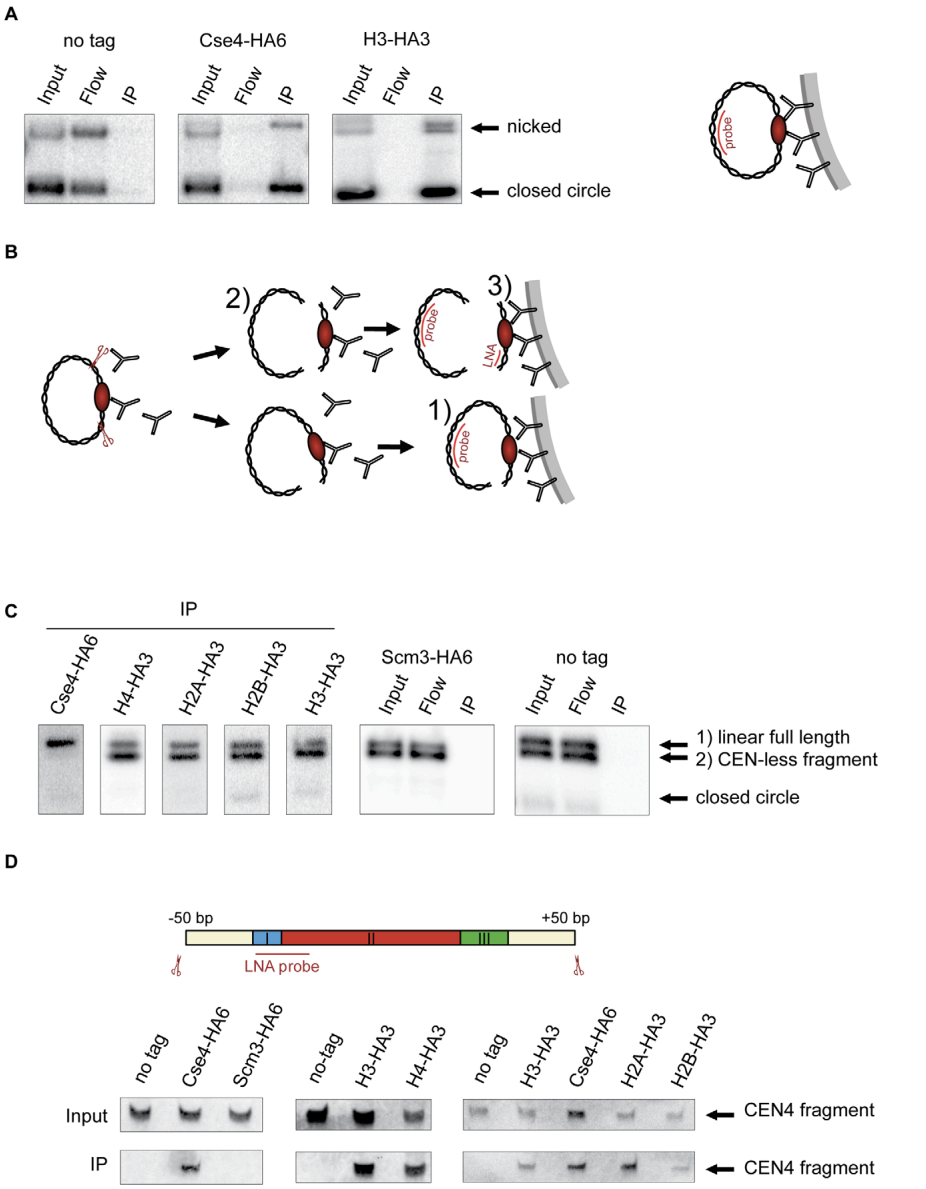
We developed new versions of ChIP to reveal the composition of the centromeric nucleosome in budding yeast. There are three main differences from conventional ChIP. First, we performed our experiments with and without the chromatin cross-linking. We reasoned that omitting cross-linking improves the accessibility of the centromeric nucleosome to antibodies and prevents potential artifacts due to the cross-linking of loosely associated proteins. However, because cross-linking prevents local re-arrangements due to nucleosomal sliding along the DNA, we also included cross-linked samples in our analysis. Second, we flanked CEN DNA by restriction sites and excised it by a specific endonuclease similar to earlier studies by [36]. Finally, analysis of the immunoprecipitated DNA was performed using methods that identify the isolated fragments by size, initially by a Southern blot with specific probes hybridizing to the excised CEN fragment. In experiments where qPCR with a specific pair of primers was used, the immunoprecipitated DNA was size-fractionated prior to PCR to preclude the detection of uncut DNA. The Biggins's laboratory recently employed a similar approach [37]. In this study, micrococcal-nuclease digested chromatin was immunoprecipitated with an anti-Cse4 antibody and analyzed by Southern blot. The results demonstrated a single Cse4 nucleosome positioned at the budding yeast centromere but did not address its composition further.

Cse4 and H3 localize to a 214 bp CEN fragment

In our initial experiments we used a small minichromosome that contained the CEN region of chromosome IV (Figure S1A). We utilized strains with HA-tagged versions of H3 and Cse4 and found that the minichromosome can be specifically co-immunoprecipitated with an anti-HA antibody even in the absence of cross-linking (Figure 1A). This result demonstrates that the minichromosome assembles conventional nucleosomes as well as a centromeric nucleosome. Next, we tested whether it is possible to digest the minichromosome in yeast cell lysate and subsequently immunoprecipitate the fragments. We constructed minichromosomes with BglII sites at different positions with respect to CEN. The digest efficiency was highly variable depending on the position of the BglII site (Figure S1B). It was previously reported that the centromeric DNA is inaccessible for the nuclease digest [33,34]. However, under our conditions it was possible to excise CEN DNA and even to cut it between CDEII and CDEIII in agreement with the previous results by [38,39].

In subsequent ChIP experiments we used a minichromosome with BglII restriction sites 50 bp upstream and downstream of CEN4 boundaries flanking a 214 bp CEN fragment. The chromatin was digested with the endonuclease BglII and immunoprecipitated with an anti-HA antibody (Figure 1B). A probe hybridizing to the *TRP1* gene located on the minichromosome outside of CEN was used for the Southern blot. Due to an incomplete chromatin digest, a linearized full-length minichromosome and a CEN-less fragment could be detected. Only the full-length linearized minichromosome co-immunoprecipitated with Cse4-HA6 while both the full-length linearized minichromosome and the CEN-less fragment were recovered with HA-tagged histones H4, H2A, H2B and H3 (Figure 1C). Therefore, although the minichromosomes assemble conventional nucleosomes along their entire length, only CEN DNA is associated with Cse4, which is in agreement with [37]. Since it was proposed recently that the Scm3 histone chaperone might replace H2A/H2B dimers in the centromeric nucleosome [11,17] we performed the minichromosome ChIP with the Scm3-HA6 strain. We could not co-immunoprecipitate the minichromosome with HA-tagged Scm3 under our conditions indicating that Scm3 is unlikely to be a part of the centromeric nucleosome (Figure 1C).

The observation that no CEN-less fragment was recovered in the Cse4-HA6 immunoprecipitation rules out lateral sliding of Cse4 nucleosome during the course of the immunoprecipitation as well as tethering of DNA fragments via protein-protein interactions, e.g., between centromeric and conventional nucleosomes in our assay. The efficiency of immunoprecipitation of the minichromosome fragments of approximately 1000 bp and longer was exceptionally high and close to 100%. When a 930 bp fragment


A
no tag
Cse4-HA6
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H2B-HA3
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CEN4 fragment

Figure 1. Composition of the centromeric nucleosome. A) The CEN-containing minichromosomes can be specifically co-immunoprecipitated with Cse4 and H3. Lysates from strains transformed with the minichromosomes 1021 (wt), 1498 (Cse4-HA6) and 1407 (H3-HA3) were incubated with anti-HA antibody and Dynabeads. DNA was eluted off the beads and separated on a 1% agarose gel. Southern blot was analyzed using a ^{32}P labeled *TRP1* probe. The map of the minichromosome is shown in Figure S1. B) Experimental setup for the immunoprecipitation of minichromosomes digested with restriction enzyme. Chromatin is digested with BglII and incubated with anti-HA antibody recognizing tagged histones and protein A Dynabeads. Minichromosome digest with BglII produces three different fragments: a linearized full-length minichromosome (1), a CEN-less fragment (2) which can be detected with *TRP1* probe and a small CEN fragment (3) which can be detected with an LNA oligonucleotide. The red ellipse is depicting the centromeric nucleosome. C) Cse4 binding is restricted to minichromosomal CEN DNA. BglII-treated chromatin of strains carrying the minichromosome with BglII restriction sites 50 bp upstream and downstream of CEN boundaries was immunoprecipitated with anti-HA antibody. The strains were 1498 (Cse4-HA6), 1577 (H4-HA3), 1576 (H2A-HA3), 1587 (H2B-HA3), 1407 (H3-HA3), 1593 (Scm3-HA6), and 1021 (wt). DNA was analyzed as in (A) with ^{32}P labeled *TRP1* probe. D) H3 is associated with the CEN DNA. Top: Scheme of the excised CEN fragment. Double-DIG labeled LNA probe for CDEI/II is indicated. Bottom: Immunoprecipitated DNA from experiments shown in (C) was separated on a 6% denaturing TBE polyacrylamide gel. Southern blot was analyzed using a double-DIG labeled LNA probe for CDEI/II. Western blots showing immunoprecipitation of the tagged proteins are shown in Figure S4A.
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from *ARS1* until position +50 downstream of CDEIII was excised, it could be depleted from yeast cell lysate with anti-HA antibodies recognizing Cse4-HA6 while virtually none of the remaining CEN-less fragment of the minichromosome could be detected on the beads (Figure S2). Considering the immediate proximity of the +50 cutting site to the centromere it is highly unlikely that there was a significant local rearrangement of nucleosomes and/or tethering of the CEN fragment to the rest of the minichromosome under our experimental conditions.

The detection of the small 214 bp CEN fragment was very inefficient using the ^{32}P-labelled probe. Therefore we employed a digoxygenin (DIG)-labeled locked nucleic acid (LNA) oligonucleotide (Figure 1D) with improved hybridization properties [40]. Using the LNA probe it was possible to detect the 214 bp fragment released from 6 pg of the minichromosome which corresponds to about 0.1% efficiency of immunoprecipitation starting with 150 ml of yeast culture in the early log phase (Figure S3). We could detect the 214 bp CEN fragment in the immunoprecipitates with Cse4, H4, H2A and H2B. Surprisingly, we reproducibly observed an interaction of H3 with the 214 bp CEN fragment using this method (Figure 1D). This was in contrast with previous studies proposing that H3 is replaced by Cse4 at the centromere [2].

We next tested whether the interaction of H3 with CEN is dependent on the cell cycle stage as it is possible that Cse4 replaces H3 at a specific point in the cell cycle. The notion that the composition of the centromeric nucleosome might vary through the cell cycle was proposed earlier [17,28]. Yeast cultures were arrested in G1-phase with alpha-factor and in G2-phase with nocodazole/benomyl (Figure S4B), and chromatin was digested with BglII to release the 214 bp CEN fragment prior to immunoprecipitation. Both H3 and Cse4, as well as H2B, were found to be associated with CEN in G1-phase and in G2-phase (Figure 2A).

Although nearly a 100% efficiency of co-immunoprecipitation of the minichromosomes with Cse4-HA6 (Figure 1A) indicated that it is unlikely to be the case, it is possible that a fraction of minichromosomes assemble a conventional nucleosome at the centromere and this would explain the association of H3 with CEN DNA in the above experiments. To address this possibility we adapted our ChIP approach to the native centromeres on the chromosomes and introduced BglII restriction sites 50 bp upstream and downstream of CEN on chromosome IV. The excised "native" 214 bp CEN4 fragment could be efficiently co-immunoprecipitated with H3-HA3 and Cse4-HA6 (Figure 2B). We conclude that both histones H3 and Cse4 localize to centromeric DNA in budding yeast.

In order to rule out the possibility that Cse4 is replaced by H3 during our immunoprecipitation procedure, we mixed yeast cell lysate of an H3-HA3 strain that does not carry minichromosomes with lysate of an untagged H3 strain carrying the minichromosomes. We could not observe any immunoprecipitation of the minichromosome with anti-HA antibody from those mixed lysates (Figure 2C). Thus there is little or no turnover of minichromosome-associated H3 in our cell lysates.

However, this experiment could not rule out local rearrangement of nucleosomes such as lateral sliding in the course of our experimental procedure, which included long incubations. Therefore we cross-linked proteins to DNA with formaldehyde prior to immunoprecipitation. Adding formaldehyde to the spheroplasts dramatically reduced the efficiency of centromeric DNA co-immunoprecipitation with either Cse4 or H3. This was partially due to the low yield of the minichromosome in the cleared lysate after centrifugation presumably because the minichromosomes were cross-linked to larger structures. However, when formaldehyde was added directly to yeast lysate the immunoprecipitation was not impeded. In order to minimize the potential rearrangement of nucleosomes after cell lysis, the duration of the restriction digest of the minichromosomes was limited to 5 minutes followed by formaldehyde addition and immunoprecipitation. We were able to efficiently co-immunoprecipitate the 214 bp CEN fragment with both Cse4 and H3 after cross-linking (Figure S5A). Therefore, it is unlikely that the detection of H3 at the CEN DNA is due to nucleosomal sliding during our experimental procedure.

A qPCR-based approach was employed to compare the efficiencies of co-immunoprecipitation of the CEN DNA with H3-HA3 and Cse4-HA6. After excision of the 214 bp CEN fragment CEN DNA was co-immunoprecipitated with Cse4-HA or H3-HA using anti-HA antibodies, eluted off the beads using SDS, size-fractionated via agarose gel-electrophoresis to separate it from full-length minichromosome and quantified using a quantitative PCR reaction. Using this procedure, we ensured that the 214 bp CEN fragment was exclusively detected since no PCR product was obtained when the restriction digest step was omitted (Figure 3A). We did not observe any significant differences in ChIP efficiencies with H3 and Cse4 when the same anti-HA antibody was used. Similar IP/input ratios were observed with and without crosslink (Figure 3B) with the CEN DNA located on a minichromosome and on the native chromosome IV flanked by restriction sites (Figure 3C). Thus we have no indication that only some centromeres are associated with H3.

Co-occupancy of the centromeric DNA by histone H3 and Cse4

The association of H3 and Cse4 with yeast centromeres can be mutually exclusive, i.e., a fraction of the centromeres are occupied by the Cse4 nucleosome while a different fraction assembles a

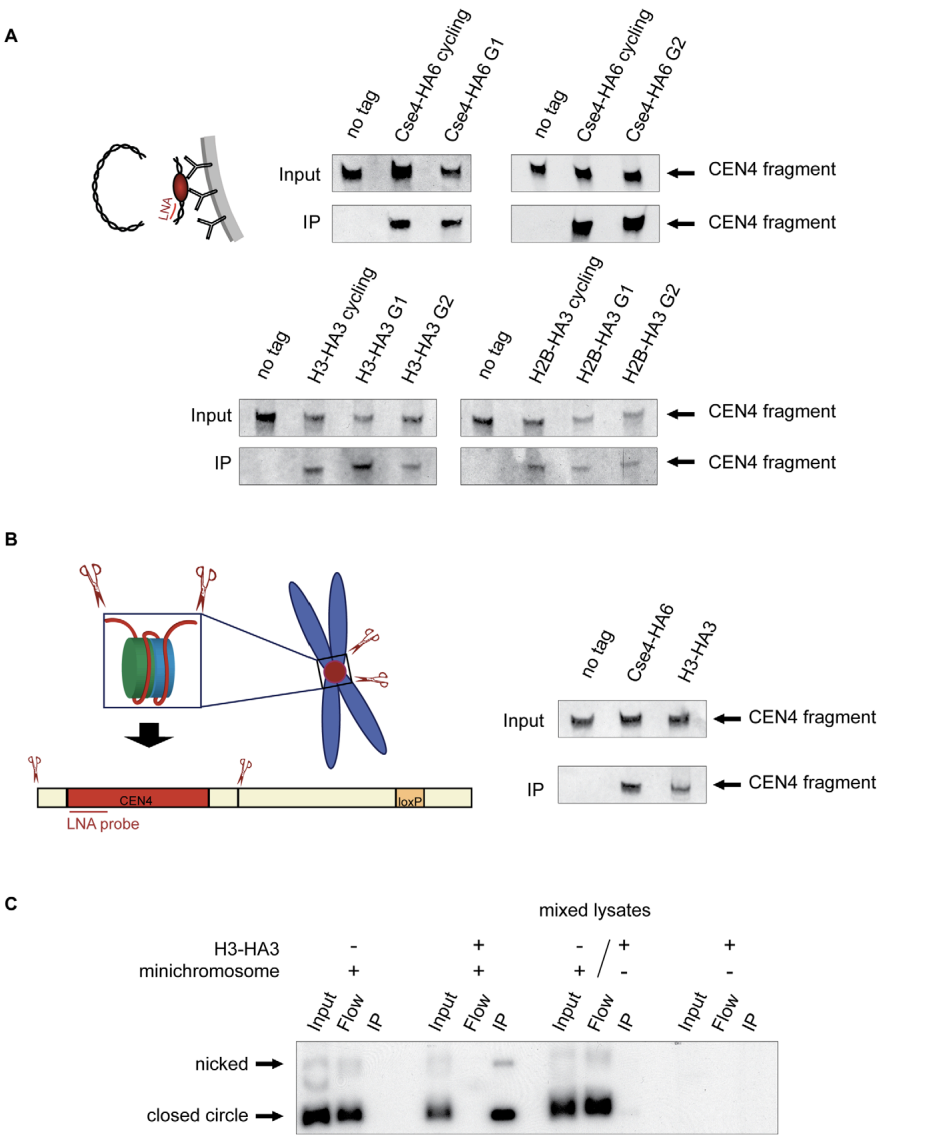

Figure 2. Histone H3 localizes to the centromeric DNA. A) H3 is associated with CEN DNA throughout the cell cycle. Strains carrying the minichromosomes with BglII restriction sites 50 bp upstream and downstream of CEN boundaries, 1498 (Cse4-HA6), 1407 (H3-HA3), and 1587 (H2B-HA3) were arrested in G1 with alpha factor and in G2 with nocodazole/benomyl. Chromatin was treated with BglII and immunoprecipitated with anti-HA antibody. DNA was eluted off the beads and resolved on a 6% denaturing TBE polyacrylamide gel. Southern blot was analyzed with a double-DIG labeled LNA probe for CDEI/II. The FACS profiles are shown in Figure S4B. B) H3 is associated with the CEN DNA on a native chromosome IV. BglII-

treated chromatin of strains with BglII sites 50 bp upstream and downstream of CEN boundaries on chromosome IV 2059 (wt), 2043 (Cse4-HA3), and 2042 (H3-HA3) was immunoprecipitated with anti-HA antibody. DNA was eluted off the beads, separated on a 6% denaturing TBE polyacrylamide gel and analyzed with a double-DIG labeled LNA probe for CDEI/II. C) Minichromosome-bound histone H3 does not turn over during the immunoprecipitation procedure. Lysates of strains 1021 (wt, carrying the minichromosome), 1407 (H3-HA, carrying the minichromosome), 1407 (H3-HA3, without the minichromosome), and mixed lysate of 1021 (wt with minichromosome) and 1407 (H3-HA3, without the minichromosome) were incubated with anti-HA antibody and Dynabeads. DNA was eluted off the beads, separated on a 1% agarose gel and analyzed using a ^{32}P labeled *TRP1* probe.
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conventional nucleosome containing H3. Alternatively, H3 and Cse4 are co-occupying the centromeric DNA at the same time. In order to distinguish between these two possibilities we performed a sequential ChIP experiment. After excision of the 214 bp CEN fragment and formaldehyde cross-linking CEN DNA was co-immunoprecipitated with Cse4-Myc using anti-Myc antibodies covalently coupled to the beads (Figure S5B and S5C), eluted off the beads using SDS, and re-immunoprecipitated with anti-HA antibodies recognizing H3-HA. The CEN DNA fragment eluted off the beads was decross-linked, size-fractionated via agarose gel-electrophoresis to separate it from uncut DNA, and quantified using a quantitative PCR reaction (Figure 3D 3F). The efficiency of the second immunoprecipitation step in this experiment was approximately 100 fold higher than the "mock" immunoprecipitation from a strain in which only Cse4 was tagged and was comparable to that of H3-HA re-immunoprecipitation in the experiment where both the first and the second steps were performed with anti-HA antibodies. Similar results were obtained when CEN DNA was excised from the minichromosome (Figure 3E) or native chromosome (Figure 3F). We conclude that H3 and Cse4 co-exist at least at some centromeres. Unfortunately, we could not perform the reverse experiment, i.e., to immunoprecipitate the CEN DNA via HA-tagged histone H3 and then re-precipitate via Myc-tagged Cse4, since we could not re-precipitate CEN DNA from Cse4-Myc strain with anti-Myc antibody in 0.1% SDS. Switching the tags was also unsuccessful since the H3-Myc6 strain was not viable.

Is the centromeric nucleosome a heterotypic octamer?

Because the length of our excised centromeric fragment (214 bp) is much shorter than would be necessary to accommodate two conventional nucleosomes (292 bp assuming no linker DNA in-between) or a conventional nucleosome and a Cse4 nucleosome (268 bp if the Cse4 nucleosome organizes only 121 bp of DNA [41]), it is plausible that the centromeric nucleosome is a heterotypic octamer with one molecule of H3 and one molecule of Cse4. If the structure of this hypothetical heterotypic nucleosome is similar to the structure of the conventional nucleosome and the CENP-A containing nucleosome [41,42], histones H3 and Cse4 are expected to form a four-helix bundle with parts of their α2 and α3 helices. In vertebrates and many other organisms the α2 helix of H3 contains a cysteine residue, C110. These cysteine residues from two histones H3 within the same nucleosome are within 6.2 Å from each other [42] and were reported to form a disulfide bond under oxidizing conditions in vitro [43]. In human CENP-A the corresponding residue is a leucine, L112, although CENP-A proteins from some other mammals, such as platypus, as well as birds and amphibians have a cysteine in this position. In the recently reported crystal structures of human CENP-A nucleosome the two leucines 112 are 4.8 5.7 Å apart [27,41], which should allow cross-linking if they are mutated to cysteines. (Figure S6A). In order to test whether a cross-link between two Cse4 molecules or between Cse4 and H3 is at all possible we co-expressed the histone fold domain of Cse4-Cys and the full-length H3-Cys in bacteria. We could observe the formation of spontaneous covalently cross-linked H3 homodimers, Cse4 homodimers and some H3/Cse4 heterodimers. The dimers were detected after denaturing SDS-electrophoresis and could be resolved by β-mercaptoethanol treatment indicating that they indeed resulted from the formation of the disulfide bond between the cysteine residues (Figure S6B).

We reasoned that disulfide bond formation between the two α2 helix cysteines would only be possible if the two histones form a four helix bundle and the ability to cross-link Cse4 and H3 would be a test of a heterotypic octamer model. Since in budding yeast neither H3 nor Cse4 contain cysteine residues, we mutated the corresponding alanine 111 and leucine 204 to cysteines. We were able to cross-link homodimers of H3-Cys in crude lysates and on isolated chromatin in the presence of 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB, Ellman's reagent), which has been reported to facilitate intermolecular disulfide bond formation between H3 histones in chicken nucleosomes [44] (Figure S7A). We could also cross-link H3-Cys histones using cysteine-specific cross-linkers, bBBr and BMOE. However, we did not observe a reproducible cross-link either between two Cse4-Cys molecules or between Cse4-Cys and H3-Cys (Figure S7B) in crude yeast lysate or isolated chromatin.

Thus we currently have no direct evidence for the presence of the heterotypic octamer at budding yeast centromeres. It is possible that the heterotypic nucleosome has a very unusual structure compared to the conventional H3-H3 nucleosome [42] or the human CENP-A-CENP-A octamer that were recently reported [27,41] and that this structure does not allow for the cysteine cross-link. It remains to be confirmed whether the cysteines can be cross-linked in the context of the fully assembled octamers.

Cse4 and histone H3 do not occupy separate sub-regions within the centromeric DNA

An alternative to the octamer is the hemisome model, which proposes a tetramer consisting of Cse4, H4, H2A and H2B histones [30,31]. Our refinement of this model will imply that in budding yeast in the immediate vicinity of the Cse4 hemisome there is either a conventional nucleosome or, possibly, an H3-containing hemisome. According to the recently reported structure, the human CENP-A-containing octamer assembled in vitro organizes 121 bp of DNA [41] while a conventional nucleosome wraps 147 bp of DNA. Thus, a Cse4 hemisome and a conventional nucleosome without any linker in-between would require approximately 207 bp which would fit with the size of our excised centromeric fragment of 214 bp. An important and testable prediction of this model is that Cse4 and histone H3 are incorporated into distinct structures, which can be potentially mapped to different stretches of DNA.

The budding yeast centromere is defined by a 125 bp sequence [45] consisting of three elements. CDEI is a non-essential 8 bp palindrome, CDEII is 78 86 bp long and is composed of 87 98% A/T, and CDEIII is a highly conserved 25 bp sequence which binds the CBF3 protein complex [46]. We conducted a series of experiments in which we tested whether Cse4 and histone H3

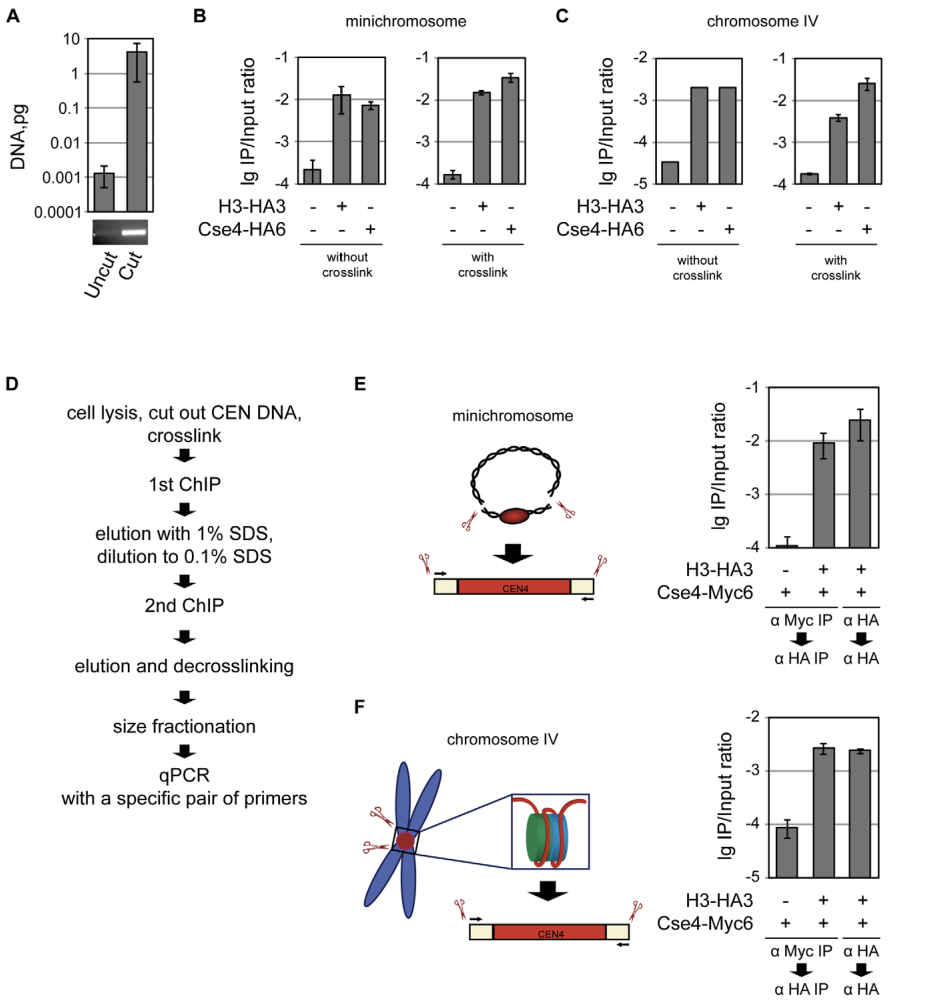

Figure 3. Co-occupancy of the centromeric DNA by histone H3 and Cse4. A) Only the 214 bp BglII CEN4 fragment and no full-length minichromosome is detected in the ChIP/qPCR assay. DNA isolated from untreated and BglII-treated lysates was size-fractionated on 2% agarose gel and analyzed by qPCR. A PCR product after 30 cycles of amplification in a conventional PCR reaction with the same primers that were used for qPCR is shown below. B) Minichromosomal CEN DNA can be co-immunoprecipitated with H3 and Cse4. BglII-treated chromatin of the strains 1021 (wt), 1407 (H3-HA3), and 1498 (Cse4-HA6) carrying the minichromosome was either not cross-linked or cross-linked with formaldehyde and immunoprecipitated with anti-HA antibody. The immunoprecipitated DNA was purified and size fractionated and subjected to qPCR analysis. C) CEN DNA of the native chromosome IV can be co-immunoprecipitatd with H3 and Cse4. BglII-treated chromatin of the strains 2059 (wt), 2042 (H3-HA3), and 2043 (Cse4-HA6) with CEN DNA of the native chromosome IV flanked with BglII was either not cross-linked or cross-linked with formaldehyde followed by immunoprecipitation as in (B). D) Flowchart of the sequential Cse4-H3 ChIP. E) Sequential ChIP of minichromosomal CEN DNA. BglII-treated chromatin of the strains 1923 (Cse4-Myc6) and 2300 (H3-HA3, Cse4-Myc6) carrying the minichromosome was cross-linked with formaldehyde and immunoprecipitated with anti-Myc or anti-HA antibody as indicated in the figure, the DNA was eluted off the beads and re-immunoprecipitated with anti-HA antibody. The immunoprecipitated DNA was purified, size fractionated on a 2% agarose gel and subjected to qPCR analysis. F) The same as in (E) but performed with the native CEN DNA. The strains, 2562 (Cse4-Myc6), and 2561 (H3-HA3, Cse4-Myc6) had CEN DNA of the native chromosome IV flanked with BglII. The bar graphs represent the average values from several independent experiments with SDs.
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associate with distinct elements within CEN DNA. It was reported earlier that *CSE4* genetically interacts with CDEI and CDEII but not with CDEIII [47] suggesting that the Cse4-containing nucleosome is localized upstream of the CDEII/CDEIII boundary. Since we were able to cut the minichromosome between CDEII and CDEIII we hoped to gain further insights in the exact localization of Cse4 with regard to CEN by using our ChIP approach. We created a minichromosome with a restriction site between CDEII and CDEIII and a restriction site outside of the CEN DNA, in *ARS1*. Using our ChIP approach we were able to co-immunoprecipitate Cse4-HA6 with both the CDEI/CDEII and the CDEIII-containing fragments (Figure 4A) suggesting that the centromeric nucleosome straddles the boundary between CDEII and CDEIII. However, an interaction with the CDEIII fragment appeared less efficient, indicating that the Cse4-containing nucleosome interacts mostly with the CDEI/CDEII region of the CEN DNA. An important corollary from this observation is that in our assay the Cse4-containing nucleosome (or hemisome) is not displaced from the CEN DNA to the edge of the 214 bp fragment.

To gain further insight into spatial distribution of H3 and Cse4-containing nucleosomes on CEN DNA we next excised a 139 bp fragment from position −50 upstream of CDEI until the CDEII/CDEIII boundary. When cross-linked, this fragment could be co-immunoprecipitated with both H3 and Cse4 (Figure 4B). This result demonstrates that H3 is present at the CDEI/II region of the centromere and/or at the preceding 50 bp of the non-centromeric DNA. Since the detection of a fragment containing CDEIII and 50 bp of DNA downstream of the CEN DNA with the LNA probe was not possible, we followed the association of histone H3 and Cse4 with CDEI/II and CDEIII elements using qPCR. Both the fragment containing CDEI/II region with upstream 50 bp and the fragment containing CDEIII region with the downstream 50 bp could be co-immunoprecipitated with HA-tagged Cse4 and histone H3 with and without crosslinking (Figure 4C). Therefore histone H3 and Cse4 appeared to be inseparable when associated with the CEN DNA implying that they are likely to be a part of one and the same structure. We would like to note that since Cse4 is capable of tethering CDEII and CDEIII fragments together (Figure 4A), the co-immunoprecipitation of the small CDEI/II and CDEIII fragments with H3 might be due to the small CDE-containing fragments maintaining the association with the large CDE-less fragment of the minichromosome throughout co-immunoprecipitation. No such tethering was observed when the complete 214 bp CEN DNA containing fragment was excised from the minichromosome (Figure 1C and Figure S2).

Discussion

Three models of the centromeric nucleosome are proposed in the literature. In the first model the centromeric nucleosome is an octamer, where Cse4/CENP-A replaces histone H3. While octameric nucleosomes with two copies of budding yeast Cse4 [48,49] or human CENP-A [41] were assembled in vitro, whether only one or both copies of H3 are replaced in vivo is not known. There is evidence from different organisms for and against either of these possibilities. In HeLa cells CENP-A released from chromatin by micrococcal nuclease digestion is still associated with histone H3 even after 2M NaCl treatment resulting in dissociation of H2A and H2B, implying heterotypic tetramers with two histones H4, one H3 and one CENP-A [4]. In contrast, in *Drosophila* S2 and Kc cells when chromatin is digested with micrococcal nuclease and CENP-A/CID is immunoprecipitated, no H3 co-purifies with CENP-A [1]. It was recently reported that *Drosophila* CENP-A/CID forms homodimers in vivo, which are unexpectedly very salt-sensitive but could be crosslinked via cysteines in the four-helix bundle after a prolonged incubation [50]. The authors did not exclude the formation of H3-CENP-A/CID heterodimers in addition to CENP-A/CID homodimers and it remains possible that different forms of CENP-A/CID nucleosomes are simultaneously present at the regional centromeres of *Drosophila* and possibly other higher eukaryotes.

In this study we demonstrate that a budding yeast centromeric DNA fragment of only 214 bp is associated in vivo with both H3 and Cse4. We can exclude a homotypic octamer with two copies of Cse4. Our experiments suggest a very intimate spatial association between the conventional histone H3 and centromeric Cse4. This association cannot be explained if the Cse4-containing centromeric nucleosome is separated from the neighboring conventional H3 nucleosomes by spacer DNA as was proposed recently [51] but rather suggests that H3 and Cse4 co-occupy the CEN DNA fragment of only 214 bp in length. We favor the Cse4-H3 heterotypic octamer model (Figure 5, model 1). This octamer appears to be resistant to cysteine cross-linking, which might be due to the reduced stability of the four-helix bundle similar to the *Drosophila* CENP-A/CID [50].

The hexamer model postulates that in budding yeast the non-histone protein Scm3 replaces H2A and H2B and the nucleosome is composed of two copies each of Scm3, CENP-A and H4 [11,17]. Although it was initially proposed that the Scm3 dimer constitutes an integral part of the centromeric hexasome [11], the recent structures of budding yeast Scm3 associated with Cse4/H4 [16,18] and human HJURP in complex with CENP-A/H4 [52,53] revealed that binding of DNA as well as the $(Cse4/H4)_2$ heterotetramer formation are incompatible with Scm3 binding. In the experiments in vitro it was demonstrated that Scm3 association with the reconstituted $(Cse4/H4)_2$ nucleosome-like particles depends on a DNA binding domain within Scm3 [17]. Our results are compatible with the view that Scm3 does not form a part of the centromeric nucleosome. Under our experimental conditions we were able to co-immunoprecipitate minichromosomes with Cse4, H4, H2A, H2B and H3 but not with Scm3, which most likely dissociated from the centromere in yeast lysate.

Finally, the hemisome model proposes a tetramer consisting of Cse4, H4, H2A and H2B histones [30 32]. According to this model, the Cse4 hemisome is positioned mostly at CDEII [20] and is expected to occupy approximately 60 bp of DNA [41]. This scenario leaves approximately 77 bp on each side of our 214 bp fragment available to accommodate the H3-containing nucleosome(s). We can speculate that a hemisome with Cse4 might, for example, be incorporated into a DNA loop between the two halves of an H3-containing octamer (Figure 5, model 3). This model might explain the tripartite organization of the budding yeast centromere that was observed in the micrococcal nuclease protection pattern [20]. Although it is technically possible that 77 bp upstream and downstream of the hypothetical centromeric hemisome are wrapped around ½ of the flanking conventional nucleosomes (Figure 5, model 4), this model will result in tethering of the excised 214 bp fragment to the rest of the minichromosome which we did not observe (Figure 1C and Figure S2) and therefore can be excluded.

More exotic models can be also considered. Two recent studies compared Cse4-GFP fluorescence in vivo to independent standards and found 3.5 6.0 [54] or even 7.6 [55] Cse4-GFP molecules per budding yeast centromere in anaphase. Even more surprisingly, in prolonged G1 arrest Cse4-GFP fluorescence was reduced more than two-fold [55]. These observations are

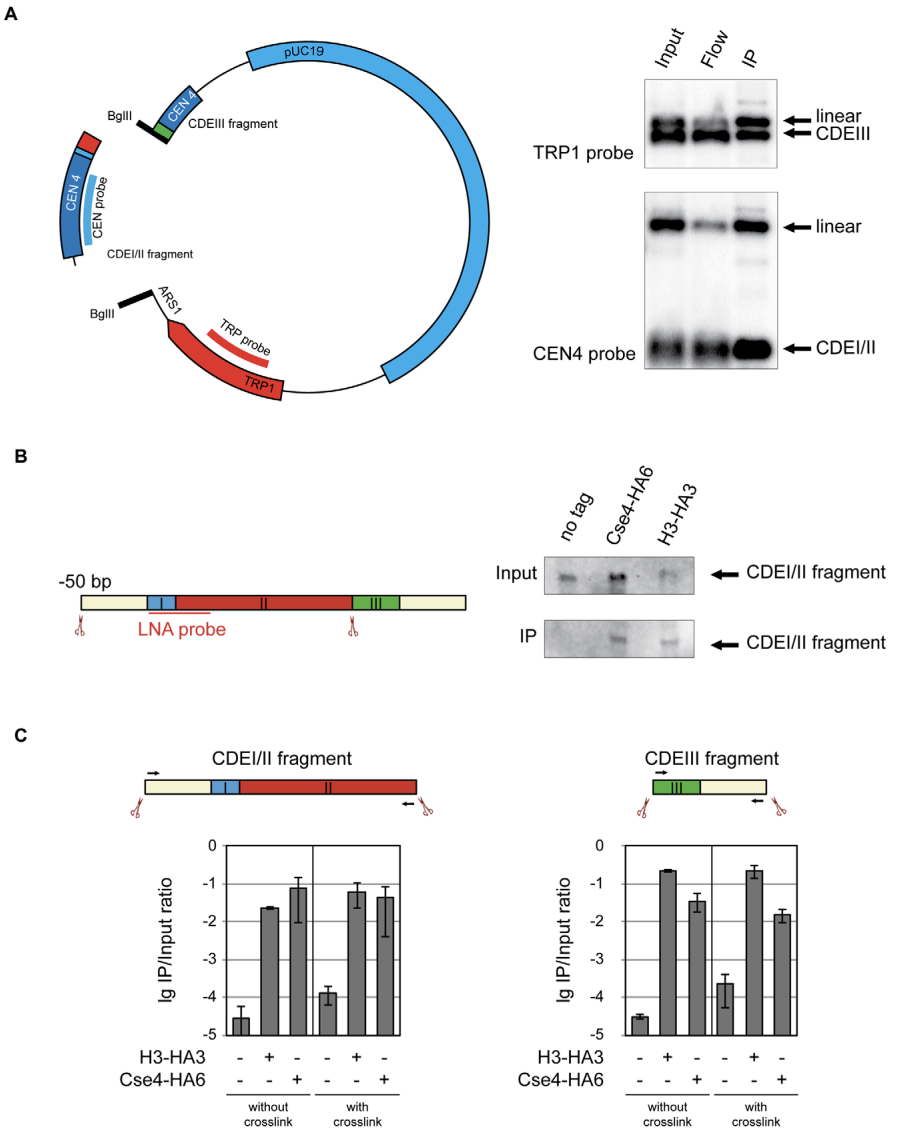

Figure 4. Cse4 association with CDEI/II and CDEIII. A) Cse4 nucleosome straddles the boundary between CDEII and CDEIII. Left: Map of the minichromosome utilized in the experiment. The construct contains 850 bp of pericentromeric sequence of chromosome IV, *TRP1* marker, *ARS1* and pUC19 sequence and has a size of 4.5 kb. There are two BglII sites: between CDEII and CDEIII in the CEN and in the *ARS1*. Right: BglII-treated chromatin of a strain 1498 (Cse4-HA6) carrying the minichromosome was immunoprecipitated with anti-HA antibody. DNA was eluted off the beads and separated on a 1% agarose gel. Southern blot was analyzed with a ^{32}P labeled probe for the pericentric CEN4 sequence (to detect the CDEI/II

containing fragment) and a ^{32}P labeled probe for the *TRP1* gene (to detect the CDEIII containing fragment). B) Both Cse4 and H3 are associated with the CDEI/II fragment. Left: Scheme of CDEI/II fragment excised from the minichromosome. Double-DIG labeled LNA probe for CDEI/II is indicated. Right: BglII-treated chromatin of strain 1498 (Cse4-HA6) and 1407 (H3-HA3) carrying the minichromosome with BglII sites between CDEII and CDEIII and 50 bp upstream of CDEI was cross-linked with formaldehyde and immunoprecipitated with anti-HA antibody. DNA was eluted off the beads and resolved on a 6% denaturing TBE polyacrylamide gel. Southern blot was analyzed with a double-DIG labeled LNA probe for CDEI/II. C) Both the CDEI/II and the CDEIII fragments can be co-immunoprecipitated with Cse4 and H3. Strains 1021 (wt), 1407 (H3-HA3), and 1498 (Cse4-HA6) carried the minichromosome where either the CDEI/II (left) or the CDEIII fragment (right) was flanked with BglII sites. BglII-treated chromatin was either not cross-linked or cross-linked with formaldehyde and immunoprecipitated with anti-HA antibody. The immunoprecipitated DNA was purified, size fractionated, and subjected to qPCR analysis. Bar graphs represent the average values from several independent experiments with SDs.
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inconsistent with the notion of a single Cse4 nucleosome at the budding yeast centromere [37]. It was proposed that the budding yeast centromere is in fact a regional centromere with additional Cse4s associated with the flanking DNA similar to the much larger centromeres of higher eukaryotes [54]. However, we could not observe any Cse4 associated with the non-centromeric part of the 2.4 kb minichromosome, which is expected to assemble 10 conventional nucleosomes. Therefore no additional Cse4 nucleosomes assemble, at least at these relatively short flanking sequences. Our results are consistent with those of [20,56] who did not detect additional Cse4 nucleosomes in centromere-flanking regions by high-resolution mapping of yeast genome. The additional Cse4 molecules at the centromere could result from Cse4 mis-incorporation which is observed in strains overexpressing Cse4 [20] and could potentially be caused by GFP-tagging. Alternatively, additional Cse4 molecules may not be incorporated into the centromeric nucleosome but are rather associated with it via protein-protein and/or protein-DNA interactions (Figure 5, model 2). In this scenario the centromeric nucleosome can be a Cse4-H3 heterotypic octamer to which more Cse4 molecules are bound. Intriguingly, when $(Cse4/H4)_2$ heterotetramers were reconstituted in the presence of Scm3 into nucleosome-like particles on a 207 bp-long high affinity nucleosome positioning DNA sequence in vitro, high molecular weight complexes possibly representing additional Cse4/H4 in loose association with the Cse4/H4/DNA complex were detected [49]. Similar complexes

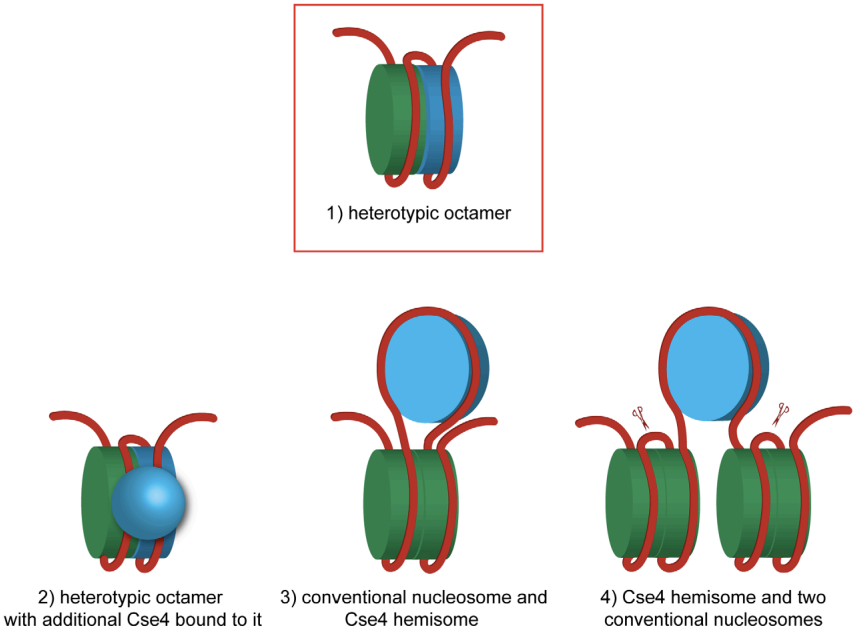

Figure 5. Models of how H3 and Cse4 can co-occupy the centromeric DNA. A heterotetramer of H3, H2A, H2B and H4 is colored in green and a heterotetramer containing Cse4 instead of H3 is blue.1) A heterotypic octamer containing both Cse4 and H3. 2) A heterotypic octamer with additional Cse4 bound to it. 3) A Cse4 hemisome incorporated in the loop of a conventional nucleosome. A DNA fragment of 207 bp is sufficient to accommodate this arrangement (without spacer DNA). 4) Two conventional nucleosomes flanking a Cse4 hemisome. The scissors indicate the BglII sites flanking the 214 bp fragment excised in our experiment. In case of model 4 this fragment would be tethered to non-centromeric DNA. The tethering was not observed in our experiments (Figure 1C). See text for discussion and additional details.
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were reported to be assembled in vitro on a 148 bp CEN3 DNA [17].

It is more than a decade now since it was proposed that H3 is replaced by the histone variant Cse4 [2]. Our results appear to contradict this well-established dogma. If Cse4 and H3 indeed co-localize to the centromeric DNA why wasn't it noticed before? We can offer the following explanation. We have noticed that in most publications reporting ChIP experiments at the budding yeast centromere, the absolute efficiency of ChIP of the CEN DNA with H3 and Cse4 is very similar and typically in the range of 1% [11,35]. The claim that only Cse4 is associated with the CEN DNA is then based on an observation that non-centromeric DNA is co-immunoprecipitated with H3 at about 5 to 10-fold higher rate than CEN DNA while almost no non-CEN DNA is found associated with Cse4 (Figure S8). We suggest that if CEN DNA were generally difficult to immunoprecipitate, for example due to cross-linking of the large number of kinetochore proteins during the in vivo cross-linking, this would explain the reduced efficiency of H3 ChIP at the centromere compared to the chromosomal arms.

Our results appear to contradict those of [35]. This group could co-immunoprecipitate differentially tagged versions of Cse4 from budding yeast but did not observe co-immunoprecipitation of tagged Cse4 and H3. However, one of the tagged Cse4s was expressed from a plasmid and Cse4 overexpression was reported to result in its ectopic incorporation genome-wide into octameric nucleosomes that were not observed in the wild type strain [20]. It remains possible that even in budding yeast there is a degree of heterogeneity in the composition of the centromeric nucleosomes among different chromosomes and that either a homotypic Cse4/Cse4 octamer or a heterotypic Cse4/H3 octamer can provide the essential function.

At this time we can only speculate at the function of H3 at the budding yeast point centromere. It is possible that the presence of two different nucleosomes (or hemisomes), one with Cse4 and one with H3 provides structural asymmetry which might form the basis for two separate surfaces, one facing the sister centromere and another providing the attachment site for the spindle microtubule.

Materials and Methods

Plasmids and strains

Generation of the minichromosome containing a 850 bp long sequence from chromosome IV encompassing CEN4 was described earlier [57,58]. A version without Tet operators was used to introduce BglII restriction sites using QuikChange Site-Directed Mutagenesis Kit (Stratagene). A SalI digest and religation was used to remove the pUC19 sequence from the final construct prior to transformation into yeast.

To introduce BglII restriction sites flanking the CEN DNA into the native chromosome IV, the region of CEN4 +/− 200 bp was cloned into the PvuII site of pOM10 (courtesy of Anne Spang) and BglII sites were introduced by mutagenesis. A yeast strain was transformed with a PCR product containing CEN4 DNA with BglII sites, marker, and a CEN flanking sequence. The BglII flanked CEN4 DNA was recombined into the endogenous locus and the marker cassette was removed with Cre recombinase [59] leaving 85 bp of the pOM10/loxP sequence 200 bp downstream of CDEIII (Figure 2B). The whole CEN4 region was sequenced.

Cse4 was tagged with HA6, Myc6 or Myc3 at an internal XbaI site as described in [2]. All other histones were tagged at the C-terminus and the second gene was either left untagged (H4) or deleted (H2A, H2B, H3). The strains are described in Table S1.

Chromatin immunoprecipitation

Yeast strains transformed with the minichromosome were grown overnight in synthetic medium without tryptophan at 30°C, were inoculated into fresh medium to a final OD_{600} of 0.2, and grown until the OD_{600} reached 1.6. For G1 arrest, yeast culture was grown from an OD_{600} of 0.05 until an OD_{600} of 0.2 and then arrested with 2 µg/ml alpha factor for 1 hour. After 1 hour, additional 1.5 µg/ml alpha factor was added followed by an additional hour of incubation. For G2/M arrest, 15 µg/ml nocodazole and 10 µg/ml benomyl were added to a yeast culture at an OD_{600} of 0.65 in YEPD medium, and cells were incubated for 1.5 hours.

Spheroplasting was carried out with lyticase (Sigma, L2524) as described in [60]. Spheroplasts were lysed for 30 min on ice in 2.5 ml of lysis buffer (25 mM HEPES/KOH [pH 8.0], 50 mM KCl, 10 mM $MgSO_4$, 10 mM Na citrate, 25 mM Na sulfite, 0.25% TritonX-100, 1 mM PMSF, 3 mM DTT, 1× complete EDTA-free protease inhibitors (Roche) and 100 µg/ml RNase A). The lysate was cleared by centrifugation at 10,000 rpm for 5 min in an Eppendorf microcentrifuge. For DNA cleavage, lysate was incubated with 1 unit/µl of BglII (NEB) for 2 hours with rotation at 4°C before adding NaCl to a final concentration of 300 mM to stop the digest. For strains with BglII sites on chromosome IV the crude lysate was incubated with BglII and cleared after 2 hours of digestion. Pre-cleared lysate (2 ml) was incubated with 25 µg of anti-HA (12CA5) antibody and 0.5 ml suspension of protein A Dynabeads (Invitrogen) overnight. Beads were washed 3 times with 1.5 ml of the lysis buffer with 300 mM NaCl. Isolated DNA was eluted off the beads two times with 250 µl of 50 mM Tris [pH 8.0], 10 mM EDTA and 1% SDS at 65°C. For cross-linked chromatin the DNA digest with BglII was performed for 5 min at 37°C, the digest was stopped by adding 300 mM NaCl and chromatin was cross-linked by adding 0.1% formaldehyde for 30 min and 125 mM glycine for 15 min on ice. The cross-linked lysate was incubated with protein A Dynabeads covalently coupled to either anti-HA (12CA5) or anti-Myc (9E11) antibody with DMP (dimethyl pimelimidate) according to the manufacturer's guidelines. For the sequential immunoprecipitation the chromatin was eluted off the beads as described above, diluted to 0.1% SDS with lysis buffer with 300 mM NaCl and immunoprecipitated with protein A Dynabeads covalently coupled to anti-HA (12CA5). The DNA was eluted off the beads as above. All the samples were adjusted to 1% SDS final concentration, extracted twice with phenol/chloroform/isoamyl alcohol (25:24:1), ethanol precipitated in the presence of 20 µg glycogen (Roche) and samples were dissolved in 20 40 µl TE. For the Southern blots detected with a ^{32}P-labelled probe specific for *TRP1* or CEN4, samples were separated on a 1% agarose gel with ethidium bromide and a capillary transfer to Hybond-N+ (GE) was carried out under neutral conditions. Blots were scanned on Personal Molecular Imager (Bio-Rad) and bands quantified with QuantityOne 4.6.7. For Southern blots detected with double-DIG labeled LNA probe (AAAGTTGATTATAAGCATGTGAC, Exiqon) samples were separated on a denaturing 6% TBE polyacrylamide gel followed by an electrophoretic transfer to Hybond-N+ at 80 V for 1 hr in 1× TBE in the Trans-Blot System (Biorad). Hybridization with DIG labeled LNA probe was performed according to instructions of DIG High Prime DNA Labeling and Detection Starter Kit II (Roche). For qPCR the samples were size fractionated on a 2% agarose gel (Certified Low Range Ultra Agarose, Bio-Rad), gel excised to separate from uncut and linear minichromosome and subjected to qPCR with the primers AGTAACTTTTGCC-TAAATCAC and TAGGTAGTGCTTTTTTTCCA for the 214 bp CEN4, TAGTAACTTTTGCCTAAATC and TAA-

TAAATAAATTATTTCATTTATGTTT for the 139 bp CDEI/II fragment, and TGTTTATGATTACCGAAACA and TTAGGTAGTGCTTTTTTTCC for the 77 bp CDEIII fragment, qPCR analysis was performed using LightCycler 480 SYBR Green I Master (Roche) according to the manufacturer's manual.

Ex vivo cross-linking of histones on chromatin

Spheroplasting was carried out using the same procedure as for ChIP. Spheroplasts were washed in 1 M sorbitol and lysed in cold reaction buffer (25 mM Sodium Phosphate [pH 7.0], 100 mM KCl, 2.5 mM $MgCl_2$, 0.25% TritonX-100) for 15 min on ice. Chromatin was pelleted using a low-speed centrifugation (4,000 rpm, 1 min) and the supernatant was discarded. The chromatin pellet was then resuspended in the reaction buffer with varying concentrations of the cross-linker. DTNB (5,5′-dithiobis-(2-nitrobenzoic acid), Sigma) was prepared as a 50 mM stock in DMSO and diluted into the reaction mixture as appropriate. Cross-linking was allowed to proceed for 1 hour on ice. The chromatin was pelleted by centrifugation and resuspended in SDS-PAGE loading dye without DTT or β-mercaptoethanol.

Protein expression in *E. coli*

Codon optimized sequences of yeast histone H3-Cys, N-terminally tagged with Avitag (Avidity), and the histone fold domain of Cse4-Cys (D150-end), N-terminally tagged with 6xHis, were cloned either together into pRSFDuet1 (Novagen) or separately, Cse4 in pETDuet1 and H3 in pRSFDuet1, transformed and expressed in BL21 (DE3) according to the manufacturer's instructions. Aliquots of bacterial culture were harvested and resuspended in SDS-PAGE loading buffer with and without β-mercaptoethanol. Samples were separated on a 15% SDS-PAGE and Western blots were analyzed with Streptavidin-HRP (Pierce) for H3-Cys and with anti-Penta-His antibody (Qiagen) for Cse4-Cys.

Supporting Information

Figure S1 Accessibility of restriction endonuclease sites in the centromeric region of the minichromosome. A) Map of the minichromosome. The construct contains 850 bp of pericentromeric sequence of chromosome IV, *TRP1* marker and *ARS1*. B) Top: Scheme of CEN4 with CDEI, CDEII and CDEIII indicated. The scissors indicate BglII sites in the different constructs. Bottom: The efficiency of a minichromosome digest at the indicated sites. DNA was isolated from BglII-treated lysates of strains carrying different minichromosomes, resolved on a 1% agarose gel and analyzed with a ^{32}P labeled *TRP1* probe.
(TIF)

Figure S2 Cse4 nucleosome remains restricted to the CEN DNA in the course of immunoprecipitation procedure. Top: Map of the minichromosome utilized in the experiment. The construct contains 850 bp of pericentromeric sequence of chromosome IV, *TRP1* marker and *ARS1*. BglII restriction sites are located 50 bp downstream of CDEIII and in *ARS1* and are indicated with scissors. Bottom: BglII-treated chromatin of a strain 1498 (Cse4-HA6) carrying the minichromomosome was immunoprecipitated with anti-HA antibody without cross-linking. A long version of the procedure with 2 hours restriction digest was used. The DNA was eluted off the beads, purified via phenol/chloroform extraction and ethanol precipitation and separated on a 1% agarose gel. Southern blot was analyzed with a *TRP1* probe to detect CEN-less fragment and a CEN4 probe hybridizing to the pericentromeric sequence to detect a fragment of the minichromosome containing CEN4.
(TIF)

Figure S3 Sensitivity of the Southern blot detection with double DIG-labeled LNA probe for CDEI/II. DNA purified from BglII-treated lysate of a strain 1021 carrying the minichromosome with BglII restriction sites 50 bp upstream and downstream of CEN4 and known quantities of the minichromosome purified from bacteria (miniprep) and digested with BglII were resolved on a 6% denaturing TBE polyacrylamide gel and analyzed by Southern blot with the LNA probe for CDEI/II.
(TIF)

Figure S4 (A) Anti-HA Western blots of samples from ChIP experiments. Input, unbound fraction and eluted beads were separated on SDS-PAGE. (B) FACS analysis of the arrested yeast cultures in the experiment in Figure 2A.
(TIF)

Figure S5 ChIP of minichromosomal and native CEN DNA fragment after formaldehyde cross-link. A) BglII-treated chromatin of the strains 1021 (wt), 1407 (H3-HA3), 1923 (Cse4-Myc6), and 2300 (H3-HA3, Cse4-Myc6) carrying the minichromosome was cross-linked with formaldehyde and immunoprecipitated with anti-HA or anti-Myc antibodies. DNA was eluted off the beads, resolved on a denaturing polyacrylamide gel and analyzed with a LNA probe for CDEI/II. B) BglII treated chromatin of the strains 1021 (wt), 1923 (Cse4-Myc6), and 2300 (H3-HA3, Cse4-Myc6) carrying the minichromosome was cross-linked with formaldehyde and immunoprecipitated with anti-Myc antibodies. Immunoprecipitated DNA was purified, size fractionated and subjected to qPCR analysis. C) Same as in (B) but performed with the native chromosome. The strains 2059 (wt), 2562 (Cse4-Myc6) and 2561 (Cse4-Myc6, H3-HA3) had CEN DNA of the native chromosome IV flanked with BglII.
(TIF)

Figure S6 H3 and Cse4 dimers can be covalently cross-linked via disulfide bonds between cysteine residues in the four-helix bundle. A) Structure of the four-helix bundle of the H3 homodimer, the CENP-A homodimer and the H3/CENP-A heterodimer. The yeast H3 histone fold domain is shown with alanine 111 and the human CENP-A histone fold domain with leucine 112 mutated to cysteines according the published nucleosome structures [41,61]. The H3/CENP-A heterodimer is modeled by superimposition of the two published homodimer structures. Sulfur atoms are depicted in yellow. B) Cysteine-containing versions of recombinant yeast full-length H3 and the histone fold domain of Cse4 were expressed together and separately in bacteria. Crude bacterial lysates were separated on SDS-PAGE and analyzed by Western blot with Streptavidin-HRP recognizing histone H3 tagged with Avitag and anti-Penta-His antibody recognizing Cse4 tagged with His6. H3/H3 homodimers, Cse4/Cse4 homodimers and H3/Cse4 heterodimers are indicated.
(TIF)

Figure S7 Cysteine-containing versions of histone H3 but not Cse4 can be cross-linked on chromatin ex vivo. Chromatin pellets were treated with DTNB to facilitate the disulfide bond formation between the cysteine side chains. Proteins were then eluted with SDS-PAGE loading buffer without β-mercaptoethanol and separated on SDS-PAGE. Western blots were analyzed with anti-HA antibody recognizing tagged H3 (A) or anti-Myc antibody recognizing tagged Cse4 (B). The strains were 1021 (wt), 1266 (H3-HA3), 1268 (H3-HA3 (A111C)) 1924 (Cse4-Myc3 (L204C)), 1949 (Cse4-Myc3 (L204C) H3 (A111C)), 1953 (Cse4-Myc3 (L204C) H3-HA3 (A111C)), and 1955 (Cse4-Myc6 (L204C) H3-HA3 (A111C)).
(TIF)

Figure S8 ChIP efficiencies of core histones and Cse4 at different locations along a chromosome. Typical ChIP efficiencies are plotted according to the data in previous reports (see main text). The ChIP efficiency of histones and Cse4 at the centromere is usually reported to be in the range of 1% whereas DNA sequences from the chromosome arms are co-immunoprecipitated with the conventional histones with about 5 10 fold higher efficiency and with Cse4 with about 5 10 fold lower efficiency.
(TIF)

Table S1 List of yeast strains.
(DOC)

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Author Contributions

Conceived and designed the experiments: BL DI. Performed the experiments: BL DI. Analyzed the data: BL DI. Wrote the paper: BL DI.

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