Epigenetic function of the amino-terminal domain of CENP-A during mitosis

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Étude des fonctions du domaine amino-terminal de CENP-A pendant la mitose

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SUMMARY OF THE THESIS

The histone variant CENP-A epigenetically marks the centromere. The presence of CENP-A at the centromeres allows the recruitment of centromeric proteins that constitute the platform for functional kinetochores.

In human cells, the NH_2-terminus of CENP-A and its phosphorylation at serine 7 in mitosis has been reported to be crucial for the progression of mitosis. However, no phosphorylation of CENP-A in other metazoan species has been described. Here, we show that the NH_2-terminus of CENP-A, but not its primary sequence, is required for mitosis in mouse embryonic fibroblast cells (MEFs). Our data show that the mitotic defects resulting from the depletion of the endogenous CENP-A can be rescued when MEFs expressing a GFP-CENP-A mutant where the NH_2-terminus of CENP-A was swapped with the phosphorylatable tail of conventional histone H3. Conversely, no rescue was observed when the two phosphorylatable serines in the H3 tail mutant were replaced with alanines. Furthermore, a non-phosphorylatable fusion mutant of CENP-A where all seven serines in the amino-tail were replaced with alanines, was also unable to rescue the mitotic phenotype of CENP-A depleted cells.

We also identified that the first three serines of the tail of CENP-A as potential sites for phosphorylation. Additionally, we were able to link the phosphorylation of CENP-A amino-tail to the proper localization of the key centromeric protein CENP-C. These results suggest that mitotic CENP-A phosphorylation is a potentially common event in metazoans essential for mitotic progression.

In the second par of this work we wanted to unambiguously tie the NH_2-terminus function of CENP-A to mitosis. To achieve this, we wanted to remove the CENP-A amino-tail only during mitosis and we devised a new method called the Hara-kiri approach in order to answer the above question in human cells. The removal of the NH_2-terminal domain of CENP-A using the Hara-kiri approach at the onset of mitosis led to increased mitotic defects in cells. Taken collectively these data show that the CENP-A NH2-terminus is required during mitosis to assure proper cell division.

Keywords
chromatin, histone variant, mitosis, phosphorylation, CENP-A

RESUME DE LA THESE

Le variant d'histone CENP-A marque épigénétiquement le centromère. La présence de CENP-A au centromère permet le recrutement de protéines centromériques qui constituent la plateforme pour l'assemblage de kinétochores fonctionnels.

Dans les cellules humaines, l'extrémité amino-terminale de CENP-A ainsi que la phosphorylation de la sérine 7, ont été signalées comme étant cruciales pour la progression de la mitose. Cependant, aucune phosphorylation de CENP-A dans d'autres espèces de métazoaires n'a été décrite. Ici, nous montrons que le domaine NH_2-terminal CENP-A, mais pas sa séquence primaire, est nécessaire pour la mitose dans les fibroblastes embryonnaires de souris (MEFs). Nos données montrent que les défauts mitotiques résultant de la déplétion de CENP-A endogène peuvent être restaurés lorsque les MEFs expriment un mutant GFP-CENP-A dont l'extrémité NH_2-terminal de CENP-A a été échangée par la queue phosphorylable de l'histone canonique H3. Inversement, dans ce même mutant, lorsque l'on remplace les deux serines phosphorylables par des résidus alanines, les défauts mitotiques persistent. En outre, le mutant de fusion non-phosphorylable de CENP-A, où les sept serines du domaine NH_2-terminal ont été remplacées par des résidus alanines, a été également incapable de restaurer le phénotype mitotique des cellules déplétées en CENP-A endogène.

Nous avons également identifié les trois premières sérines de la queue de CENP-A comme sites potentiels de phosphorylation. De plus, nos résultats montrent que l'absence de phosphorylation du domaine amino-terminal conduit à la délocalisation de la protéine centromérique CENP-C. Ces résultats suggèrent que la phosphorylation mitotique de CENP-A est un événement potentiellement fréquent chez les métazoaires et essentiel à la progression mitotique.

Dans la seconde partie de ce travail, nous avons voulu lier sans ambiguïté la fonction du domaine NH_2-terminal du CENP-A à la mitose. Nous avons conçu une nouvelle méthode, appelée approche Hara-kiri, pour pouvoir éliminer le domaine NH_2-terminal seulement pendant la mitose. Ceci afin de répondre à la question ci-dessus dans les cellules humaines. L'élimination du domaine NH_2-terminal du CENP-A en utilisant l'approche Hara-kiri en début de mitose a conduit à une augmentation des défauts mitotiques dans les cellules. Prises collectivement, ces données montrent que le domaine

NH_2-terminal CENP-A est nécessaire pendant la mitose afin d'assurer le bon déroulement de la division cellulaire.

Mots-clés
chromatine, variant d'histone, mitose, phosphorylation, CENP-A

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Defne DALKARA

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PART I INTRODUCTION

1. Chromatin

In eukaryotes DNA that is approximately 2m must be packaged in order to fit into a nucleus that is only 5 to 10 μm. To achieve this DNA of cells is tightly bound to small basic proteins called *histones*, resulting in a complex nucleoproteic structure known as *chromatin*.

The term chromatin was first used in 1881 by W. Flemming who had observed the presence of thread like structures within the nucleus that had high affinity for dyes and thus named these structures “chroma”tin from the Greek word for colour. Three years later A. Kossel used for the first time the term histone to describe the basic proteins that resulted from acid extraction of nuclei. Nevertheless the discovery of DNA as the source of genetic information was to come much later in 1944 (Olins and Olins, 2003).

1.1. The Nucleosome

The basic unit of chromatin is the nucleosome. The nucleosome core particle (NCP) is 147bp of approximately 1.65 turns of super-helical DNA wrapped around a histone octamer. Linker protein histone H1, binds to the 10 to 90bp long linker DNA located at the entry/exit sites of the nucleosome core and the resulting structure is called the nucleosome (Luger et al., 1997).

1.1.1. Histones

Histones are small, highly basic proteins found in all eukaryotic cells. They are primordial for cell viability and are highly conserved during evolution from yeast to humans (Kim et al., 1988). Histones can be grouped into five families: H1, H2A, H2B, H3 and H4. Histones within the H1 family are called linker histones and differ from core histones that constitute the nucleosome core particle.

Genes encoding for conventional histones are generally present in multiple copies in the genome and are organized in clusters, for example in humans chromosomes 6 contains 55 histone genes and chromosome 1 contains nine. Similarly in mouse there is a large cluster on chromosome 13 with 51 genes and two smaller clusters on chromosome 3 and 11 (Marzluff et al., 2002). These genes exhibit atypical features for eukaryotic genes, such as the absence of introns and the derived mRNAs that end with a stem-loop structure instead of a poly-adenylation signal. It is also important to note that their transcription is

regulated to ensure that the mRNA levels increase dramatically during the S phase and fall sharply as soon as the DNA is replicated, restricting the expression of conventional histones to the S phase. In contrast, other subtypes whose expression is not coupled to replication are collectively called histone variants (Osley, 1991).

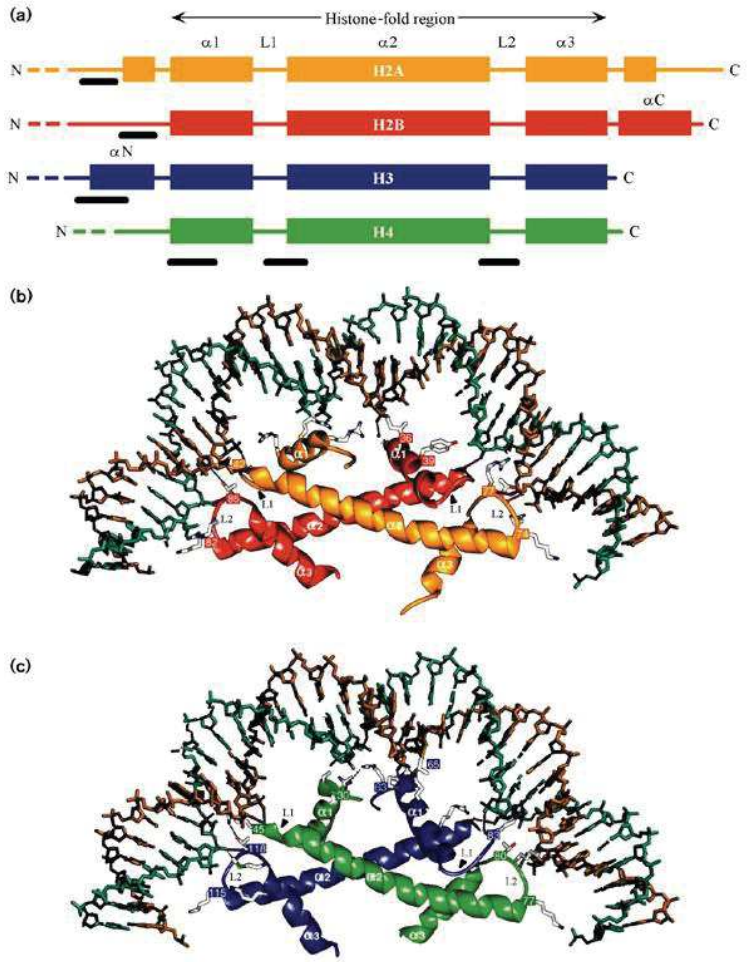


Figure 1.1 Histone families and histone fold interactions. **a)** Schematic view of the secondary structure of the histones illustrating the central histone fold domain. Interaction of the histone-fold regions of the **b)** H2A–H2B dimer and **c)** H3–H4 dimer with approximately three turns of DNA. (adopted from Dutnall et al, 1997)

Core histones share a common overall structure organized into two domains (Figure 1.1a): a globular domain that contains the highly conserved histone fold domain and unstructured terminal domains, or tails, protruding out of the nucleosome core particle. (Arents et al., 1995.) The histone fold domain is an approximately 70 amino acid long region that is comprised of one larger central alpha helix (α2) separated by loops on each side from smaller alpha helixes (α1 and α3) and enables histone dimerization important for nucleosome formation via head to tail interaction of the histone fold domains (Figure 1.1b). Histone tails are less structured and poorly conserved domains, they differ in size

and presence of random coil elements within the histone families and are highly diverse (Figure 1.1a) (Luger et al., 1997; Sandman et al., 1990).

Linker histones are the most diverse family of histones, they are very rich in lysine and differ from core histones as they lack the histone fold domain. H1 family histones typically have a three domain structure: a highly conserved central globular domain (Albig et al., 1997), a short unstructured amino tail (20aa) and a long lysine rich unstructured carboxy terminal domain (100aa)(Hartman et al., 1977). As mentioned before histones H1 bind to linker DNA in the nucleosome for stabilization but are also more dynamic within chromatin. Partial depletion of H1 is not essential for survival in mice and other organisms but plays a crucial role in chromatin state and organization (Khochbin, 2001; Roque et al., 2005; Misteli et al., 2000; Bustin et al., 2005). Whereas simultaneous depletion of three somatic H1 subtypes was lethal in mice (Bednar et al., 2016).

1.1.2. Nucleosome Core Particle and the Chromatosome

The nucleosome core particle (NCP) is the crystalized part of the nucleosome that protects DNA and hence what remains after nuclease digestion. Histones are assembled in pairs of heterodimers H2A-H2B and H3-H4 that interacts with their histone fold domains through head to tail hydrophobic interactions. Both H3-H4 dimers assemble by an interaction between the histone H3's α2 and α3 helices, forming a tetramer $(H3–H4)_2$. The H2A-H2B dimers then bind to each side of the tetramer and complete the octamer formation (Figure 1.2). Though the highly basic structure of histones renders the octamer unstable, stability is obtained by DNA binding or high salt concentration. DNA bends around the histone octamer due to charge neutralization of the acidic DNA phosphate groups, hydrophobic interactions and by hydrogen bound formation between main-chain amide groups and phosphate oxygen atoms (Luger et al., 1997).

The chromatosome is the structure that results from histone H1 binding to the NCP. Though there's multiple views on where within the nucleosome H1 binds, the widely accepted one is that it binds via its globular domain to the dyad of the nucleosome and its lysine rich carboxy-terminal tail interacts with linker DNA from each side of the nucleosome (Shaytan et al., 2015; Shaytan et al., 2016).

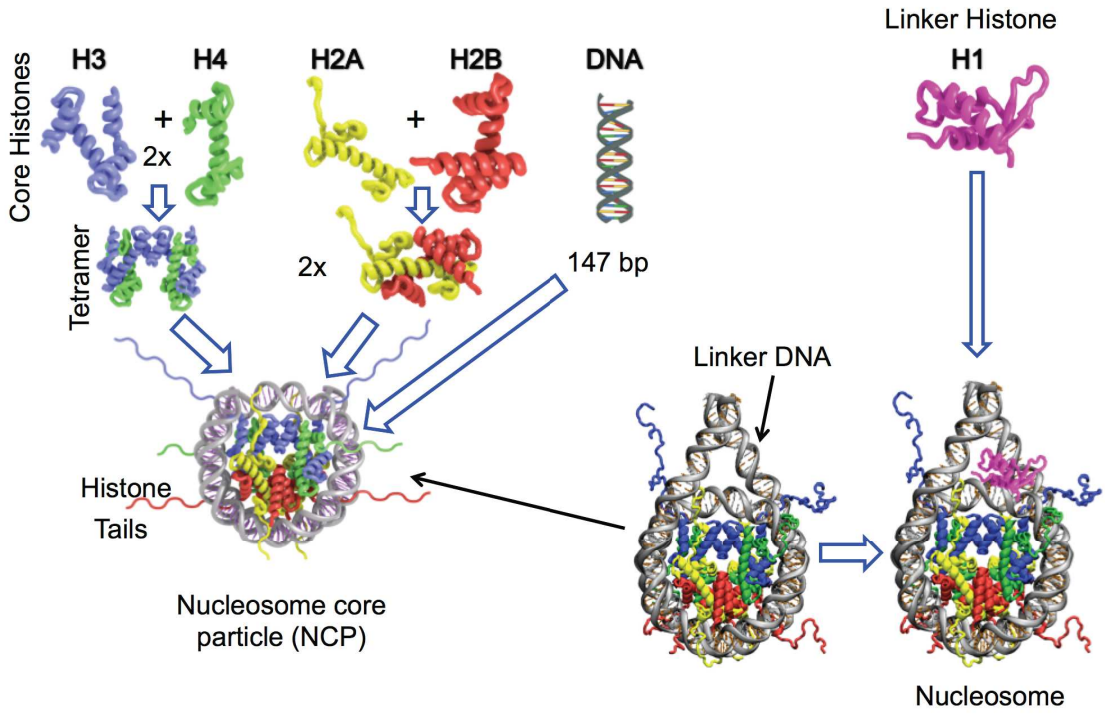


Figure 1.2 Nucleosome assembly: Histone assembly forms the NCP and is proceeded by H1 binding.

1.2. Chromatin Structure and Organization

1.2.1. Chromatin Higher-order Structures

Chromatin in cells can be organized differently depending on the level of compaction. The most relaxed conformation is called the nucleosome filament in which the succession of nucleosomes connected by linker DNA, forms an 11-nm wide linear structure, resembling "beads on a sting " (Figure 1.4). This structure is observed under low salt conditions by electron microscopy (EM) on both endogenous and reconstituted chromatin. However, this primary structure is not initially found to be favored under physiological conditions (Thoma et al., 1979; Hansen, 2002; Horowitz-Scherer and Woodcock 2006), in fact in the presence of physiological salt concentrations, linear chromatin condenses into a helical rearrangement of nucleosomes, stabilized by linker histones and known as chromatin secondary structure or the 30-nm fiber (Woodcock and Dimitrov, 2001).

Chromatin organization and nucleosome arrangement is difficult to study and remains a controversial subject. Early EM studies of native chromatin fibers proposed two models for the 30-nm fiber. The one-start helix also known as the solenoid model consists of adjacent nucleosomes connected consecutively by bent linker DNA that fallows a helical trajectory with 6 to 8 nucleosomes per turn (Widom et al., 1985). The two-start helix model is where straight linker DNA connects adjacent nucleosomes and the two

nucleosome arrays are assembled in a zig-zag manner as nucleosome cores are helically arranged (Woodcock and Ghosh, 2010; Williams et al., 1986). The two-start model is divided to two models named the helical ribbon model and the cross-linker model (Figure 1.3) (Dorgio et al., 2004).

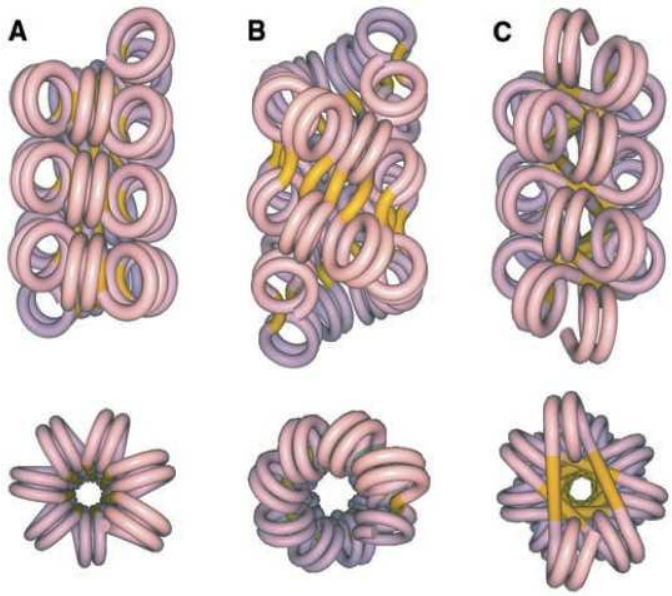


Figure 1.3 Proposed models for the 30-nm fiber organization. **a)** one-start solenoidal, b) two-start cross-linked , and c) two-start helical ribbon. Upper views have the fiber axis running vertically; lower views are down the fiber axis. Linker DNA running between cores showed in yellow. (Adopted from Dorigo et al., 2004)

Further levels of chromatin compaction do exist, the most obvious being the maximum level of chromatin compaction achieved by the metaphase chromosome. Examples to chromatin tertiary structures are chromatin loops and other higher structures found in both metaphase chromosomes and specialized regions of interphase chromosomes, such as gene enhancers and insulators (Fraser and Grosveld 1998; Woodcock and Dimitrov 2001; Woodcock and Ghosh, 2010). However the lack of definitive information about chromatin secondary structure complicates inquiries to bona fide structures above the 30-nm fiber, in fact more recent studies suggest a paradigm shift. Small-angle X-ray scattering (SAXS) experiments were unable to find repetitive structures beyond the 10-nm fiber in isolated nuclei (Joti et al., 2012) or in mitotic chromosomes (Nishino et al., 2012). Cryo-electron microscopy studies of interphase (Bouchet-Marquis et al., 2006; Gan et al., 2013) and mitotic chromosomes (Eltsov et al., 2008), and electron spectroscopic imaging studies of mouse cells (Fussner et al., 2012), observed folded 10-nm fibers but no 30-nm fibers. Chromosome conformation capture experiments describe structures within interphase chromosomes of varying size (0.1-1Mb in size depending on species), as “topologically

associating domains" (TADs) (Dixon et al., 2012; Nora et al., 2012; Sexton et al., 2012; Dekker et al., 2013; Rao et al., 2014; Eagen et al., 2015).

In light of these findings an alternative model has been proposed in which chromosomes are assembled through long-range interactions, so large oligomers are not assemblies of the 30-nm chromatin fibers, but rather are proposed to be an interdigitated polymer-melt like structure deriving from 11-nm nucleosomal arrays (Figure 1.4) (Maeshima et al., 2016).

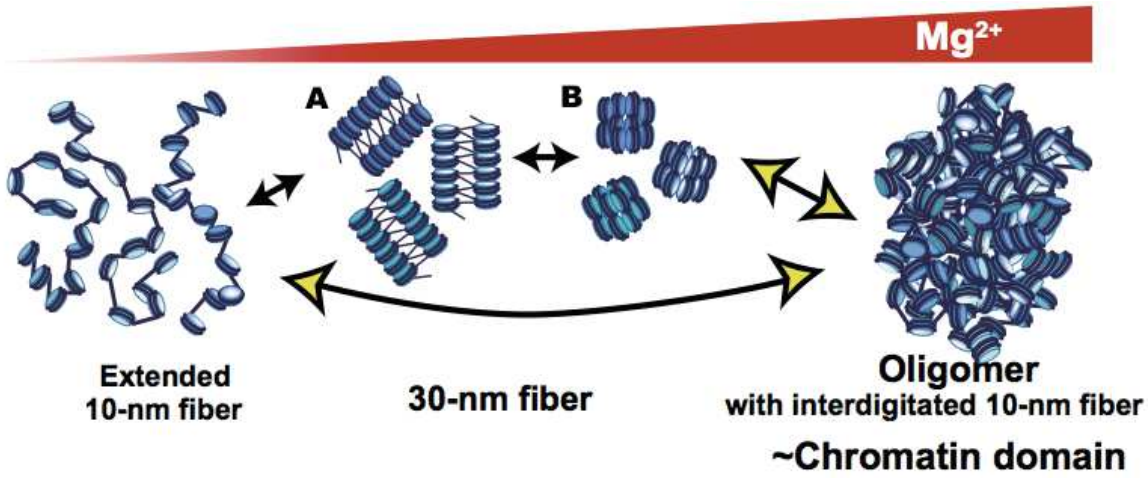


Figure 1.4 Alternative model: In 1–2 mM Mg^{2+}, the nucleosomal array folds into a folded 30-nm chromatin fiber structure. With further increases in Mg^{2+}, the nucleosome arrays assemble into supramolecular oligomers rather then the 30-nm chromatin fibers. (adopted from Maeshima et al., 2016)

1.2.2. The Metaphase Chromosome

At the beginning of mitosis, chromatin adopts a conformation of maximum compaction that is the mitotic chromosome. The typical mitotic chromosome is X-shaped with two parallel chromatids held in proximity to each other by cohesins and role of condensin. The main functional elements of the chromosome are: the telomeres that protect the DNA at the ends of each chromatid and the centromeres, on which the kinetochore forms and constitutes the anchoring points for microtubules that mobilize chromatids during mitosis. The longest human chromosome is about 10 microns long and a little less than 2 μm wide and contains 250Mb of DNA in each chromatid (Marko, 2008).

Consistent with the uncertainty that surrounds chromatin higher order structures, the structure and the formation of mitotic chromosomes are not well known and widely discussed. The existing models can be loosely subdivided into three groups: hierarchical models of increasingly thicker coiled or looped fibers, loops-on-a-scaffold models and network models, which describe mitotic chromosomes as highly cross-linked gels.

The hierarchical folding type models suggest that the mitotic chromosome results from several levels of successive folding of 30 nm chromatin wrapping into thicker fibers which in turn are wound in the upper level of fiber subsequently coil to form thicker condensed chromatids (Belmont et al., 1987). The structure as strictly ordered hierarchy of increasing levels of coiling was criticized for being overly simplistic, the Belmont laboratory showed that mitotic chromosomes are folded to varying thickness and as an irregularly condensed fiber. They proposed an axial glue model where the chromatin fibers folds hierarchically, but without strict order around a longitudinal central axis enriched in codensins (Kireeva et al., 2004).

The other model is the radial loop/scaffold model, where analysis after histone removal from mitotic chromosomes revealed chromatin loops that emanate from a dense axial structure of average of ~80 kb and consists of condensins and topoisomerase II. This loop-axis structure can then coil to further shorten the chromosome (Boy de la Tour et al., 1988; Maeshima et al., 2003).

The network model was proposed by Poirier and Marko after they performed biophysical measurements by stretching chromosomes using micropipettes and applying restriction enzymes with different cutting frequencies to chromosomes prior to stretching. They observed that chromosomes can return to original length after being stretched up to ~5 fold and postulated that mitotic chromosomes consist of network or gel in which individual chromatin fibers are connected by crosslinking elements, presumably proteins distanced by 10 to 20 kb (Poirier et al., 2002a; Poirier et al., 2002b).

More recently Job Dekkers lab applied chromosome conformation capture methods, 5C and Hi-C to reassess some of these different models. Their findings revealed that contrary to interphase chromosomes, mitotic chromosomes don't reveal any cell type-specific nor any locus-specific features and found that metaphase Hi-C data is mostly inconsistent with classic hierarchical models. In turn, they describe metaphase organization as a two-stage process: linear compaction by consecutive chromatin loops, potentially generated by cohesin and condensin complexes, followed by axial compression (Naumova et al., 2013).

Chromatin Functional Organization

Euchromatin and Heterochromatin

When interphasic nuclei were observed by electron microscopy, two distinct functional states were assigned to chromatin: *euchromatin*, corresponding to the loose areas

associated with active transcription of genes, and *heterochromatin*, corresponding to regions of compact chromatin that is often transcriptionally inactive.

Euchromatin regions are relaxed and transcriptionally active during interphase, during replication have priority and only get compacted during mitosis. This compaction coincides with the cessation of mRNA synthesis during cell division (Santos-Rosa et al., 2002).

Heterochromatin protein HP1 is essential for heterochromatin formation, and heterochromatin can be defined by two separate states: constitutive and facultative. Constitutive heterochromatin is highly condensed, maintained and is often enriched in repetitive, late replicating DNA sequences and is poor in genes, such as those present in the centromeric, pericentromeric and subtelomeric regions (Greval et al., 2007).

Facultative heterochromatin state is more flexible, decondensation can occur outside of the S-phase and these regions can become transcriptionally active. Facultative heterochromatin formation is regulated by proteins such as Polycomb-group proteins and non-coding RNAs such as Xist. Xist RNA plays an essential role in X chromosome inactivation in mammalian females and is a good example of facultative heterochromatin function. Another interesting feature of facultative heterochromatin is that it's capable of spreading to neighboring chromatin regions (Hines et al., 2009; Trojer and Reinberg, 2007; Greval et al., 2007).

Heterochromatin is often enriched at the nuclear periphery, this preferential association with the nuclear lamina (lamin A, B and C) has been widely documented, these regions are called lamina-associated domains (LADs), typically 0.1-1 Mb in size (Figure 1.5) (Pickersgill et al., 2006; Guelen et al., 2008; Kind and van Steensel, 2010).

Chromosome Territories

Chromosome territories refer to the space occupied by chromosomes during interphase, although they don't have a specific address within cell, large gene-poor chromosomes are more likely to located near the nuclear periphery, whereas small gene-rich chromosomes are located more internally. Chromosome territories don't have specific inner compartmentalization regarding active genes within the territory, in fact it has been shown that in some gene dense regions such as the HOX gene cluster, chromatin loops protrude from the territory towards transcription factories (Figure 1.5) (Cremer and Cremer, 2010).

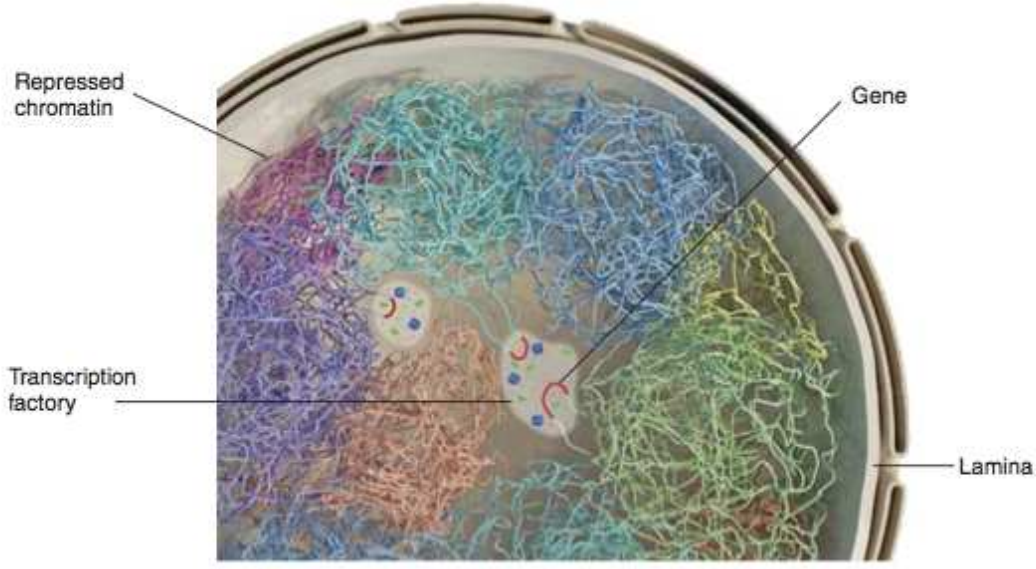


Figure 1.5 Schematic representation of the nucleus, different colors mark separate chromosome territories. (Adopted from Dekker, 2015)

1.1. Chromatin Dynamics

Chromatin organization and structure is important for living cells but the highly compact state of chromatin can render the genome relatively inaccessible to protein machineries and these structures alone are not sufficient to meet the constantly alternating physiological and metabolic needs of eukaryotic organisms. Chromatin must be able to alternate freely between structural states and hence should be a highly dynamic structure that can reorganize and assure dynamic cellular processes to modulate chromatin structure and increase genome availability to regulatory proteins. Orchestrating such dynamics is complex, partial or complete remodeling of the nucleosomes allows access to molecular machineries of transcription, replication and DNA repair. Three mechanisms ensure this dynamism: chromatin modifiers, post-translational modifications of histones and incorporation of histone variants.

1.2.3. Chromatin Remodelers

Chromatin remodelers alter chromatin state in an ATP dependent manner by moving, ejecting, reassembling or restructuring nucleosomes. Based on their domain structures there are four families of remodelers: the SWI/SNF family, the ISW1 family, the CHD family and the INO80 family. All four families share some basic properties; a high affinity for nucleosomes, histone modification recognition sites, DNA dependent ATPase domains

capable of undoing DNA/histone interactions and finally domains and/or proteins for interaction with other chromatin or transcription factors. (Clapier and Cairns, 2009).

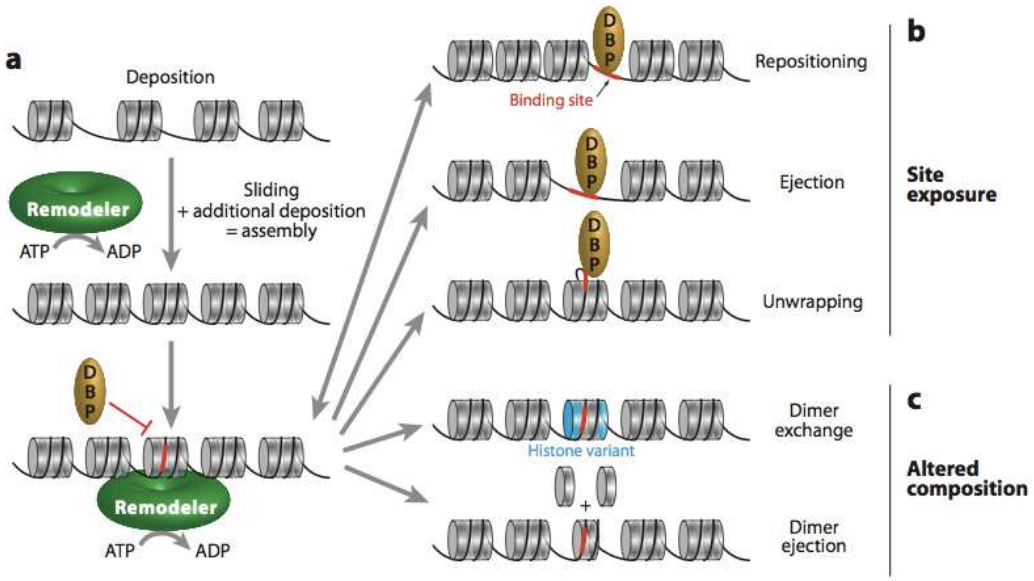


Figure 1.6 Different outcomes of chromatin remodeling. Remodelers can move already incorporated histone octamers, generating room for additional deposition **a)** Remodeler activity on a nucleosome array can lead to separate outcomes: **b)** site exposure, where a previously inaccessible site (red) for a DNA-binding protein (DBP) is revealed by nucleosome sliding (repositioning), or nucleosome eviction (ejection), or localized unwrapping, and **c)** altered composition, where the actual content of the nucleosome is altered by dimer replacement or ejection. (Adopted from Clapier and Cairns, 2009)

The SWI/SNF (switching defective/sucrose nonfermenting) family remodelers have multiple function and activities involving nucleosome sliding and eviction but are not implicated in chromatin assembly (Mohrmann and Verrijzer, 2005). The ISWI (imitation switch) family remodelers modify nucleosome spacing and assist chromatin assembly dually where they can facilitate transcriptional activation or repression. (Corona and Tamkun, 2004). The CHD (chromodomain, helicase, DNA binding) family remodelers can promote or repress transcription by sliding and ejecting nucleosomes, this variability may come from chromodomain diversity (Marfella et al., 2007). The INO80 (inositol requiring 80) family remodelers have diverse functions, including promoting transcriptional activation and DNA repair and histone dimer replacement (Figure 1.6) (Bao and Shen, 2007).

1.2.4. Histone Posttranslational Modifications

Histones are the target of a large number of post-translational modifications (PTMs), these changes are associated with processes such as transcription, DNA repair or chromatin condensation. Modifications of the amino-tails were amongst the first observed and more extensively studied. The presence of one or more modified histones can alter the properties of nucleosomes but (Mutskov et al., 1998) it has been postulated that post-translational modifications of histones exert their effects not so much by altering the nucleosome but rather by the recruiting specific proteins. A further step of this model assumes that a combination of modifications rather than individual events recruit distinct proteins, proposing a sort of "histone code" (Strahl and Allis, 2000). This hypothesis remains widely debated, including lack of a clear causal link between post-translational modifications and the activation or repression of transcription (Henikoff and Shilatifard, 2011).

Although most previously known histone modifications were on the amino-tails of histones, new modifications have been located in the structured core and these modifications are on the histone-DNA interphase. These lateral surface modifications may in fact alter the free energy of histone-DNA interactions and hence nucleosome mobility (Cosgrove et al., 2004, Lawrence et al., 2016).

Principle Histone Modifications

Histone Acetylation

Histone acetylation is a dynamic process executed by two families of enzymes with antagonistic functions, histone acetyltransferases (HATs) catalyze the transfer of an acetyl group from acetyl CoA to the ε-amino terminal group of lysine residues, whereas histone deacetylases (HDACs) catalyze lysine de-acetylation where acetylated lysines are recognized via their bromodomain and are removed (Dhalluin et al., 1999; Jacobson et al., 2000).

There are two classes of HATs: type A HATs, are diverse, localized in the nucleus and are involved in histone acetylation within chromatin. Type B HATs are located in the cytoplasm and acetylate newly synthesized histones before these histones are deposited into chromatin (Sterner and Berger, 2000). Cytoplasmic acetylations can be needed for H4 deposition such as H4K5 or necessary due potential lack of access to structured domains once deposited such as with H4K91 acetylation (Ye et al., 2005).

HDACs are most frequently associated with transcriptional repression, deacetylation stabilizes chromatin structure and histone hypoacetylation is associated with repressed chromatin state (Grunstein, 1997; Katan-Khaykovich and Struhl, 2002). Histone acetylations also play a critical role in apoptosis, DNA repair and VDJ recombination (Kouzaride, 2007).

Histone Methylation

Histone methylation is the addition of one to three methyl groups on to the side chains of lysine and arginine residues. Lysine methylation is catalyzed by histone (lysine) methyltransferases (HKMT) and arginine methylation is catalyzed by protein arginine methyltransferases (PRMT). Contrary to acetylation and phosphorylation, histone methylation does not alter the charge of the histone. Each enzyme generally targets a specific residue and the impact of said methylation varies depending on residue that has been modified and the overall number of methyl groups transferred on said residue (Lachner and Jenuwein 2002; Lachner et al., 2003). Three lysines, in particular, are associated with activation of transcription when they are methylated: H3K4, H3K36 and H3K79; conversely, di- and tri-methylation of H3K9 and H3K27 is frequently associated with transcriptional repression and is a hallmark of heterochromatin, as they are recognized by HP1 and by the polycomb repressive complex respectively (Bannister et al., 2001). More so, H4K20me1 is associated with active transcription, H4K20me2 with DNA damage response and H4K20me3 with transcriptional repression when present at promoters (Wang et al., 2008; Botuyan et al., 2006).

For a long time it was thought that histone methylation was irreversible, the identification of the amine oxidase LSD1 (a.k.a KDM1) (Allis, 2007) as a histone demethylase changed this perception (Shi et al., 2004). Currently there are many demethylases that have been identified and grouped into six families KDM1 to KDM6. For instance, certain demethylases containing the Jumonji catalytic domains, can demethylate very stable trimethylated lysines as well as methylated arginines (Cloos et al., 2008).

Histone Phosphorylation

Phosphorylation is the addition of a phosphate group on a serine, threonine or tyrosine residue. Dedicated kinases transfer the phosphate group from ATP to the hydroxyl group of the amino acid side-chain, this introduces a negative charge to the histone. In chromatin,

phosphorylation seems to occupy a less prominent place when compared to acetylation or methylation. However lately, phosphorylation is being studied and linked to more functions (Table 1: Banerjee et al., 2011). Perhaps the most known phosphorylation event is that of H3S10 and to a lesser extent that of H3S28, both involved in chromosome condensation at the beginning of mitosis, mediated by Aurora kinases. (Hans and Dimitrov, 2001). Furthermore, in mammals phosphorylation at S139 of H2A.X, a variant of histone H2A, is associated with the repair of double-strand DNA breaks (Rogakou et al., 1998).

TABLE 1. Histone residues that are phosphorylated[a]

Histone	Residue	Organism	Kinase(s)	Protein(s) recognizing (bound or released) the modification	Function(s)
H2A	Serine 139 (H2AX)	Mammals	ATR, ATM, DNA-PK, RSK2, MSK1	DSB-sensing proteins (MRN, MDC1, P53BP1), AP1	DNA repair, decreased EGF-mediated cellular transformation, apoptosis
	Tyrosine 142 (H2AX)	Mammals	WSTF	ND	Decision between cell survival and apoptosis
	Serine 1	Mammals	MSK	ND	Inhibition of transcription
	Threonine 119	Mammals	NHK-1, Aurora B	PTB domain-containing protein Fe65	Regulation of chromatin structure and function during mitosis
	Serine 121	Fission yeast	Bub1	Shugoshin	Maintaining chromosomal homeostasis by recruiting shugoshin at sister kinetochore
	Serine 129	Yeast	ATM-related kinase Mec1 and Tel1	NuA4, SWR1, INO80, cohesin	DSB repair
H2B	Serine 10	Yeast	Ste20	ND	Apoptosis
	Serine 14	Various vertebrates	MstI	ND	Apoptosis
	Serine 32	Mammals	Protein kinase C	ND	Possible involvement in apoptosis-related nucleosomal DNA fragmentation
	Serine 33	*Drosophila*	CTK-TAF1	ND	Transcriptional regulation
	Serine 36	Mammals	AMPK	ND	Direct transcriptional and chromatin regulatory pathways leading to cellular response to stress
H3	Threonine 3	Mammals	Haspin	Survivin	Correct localization of the CPC at the centromere
	Threonine 6	Mammals	$PKC\beta_1$	ND	Androgen-dependent H3T6 phosphorylation prevents LSD1-mediated H3K4 demethylation, maintaining hormone-dependent gene activation
	Serine 10	Yeast, mammals	Snf1, IpL1 (yeast), Aurora B (mammals), MSK1/2, IKKα, PKB/Akt, Rsk2, PIM1	HP1, SRp20, ASF/SF2, 14-3-3	Help in chromosome condensation during mitosis and meiosis; roles in transcription of certain genes
	Threonine 11	Mammals	Chk1, PRK1, Dlk/Zip kinase	GCN5	Transcriptional activation of certain genes; probable role at centromeres during mitosis
	Serine 28	Mammals	Aurora B, MSK1/2	Polycomb silencing complex	Help in chromosome condensation during mitosis and meiosis; roles in transcription of certain genes
	Tyrosine 41	Mammals	JAK2	HP1α	Role in differentiation related to hematopoiesis
	Threonine 45	Mammals, budding yeast	Protein kinase C, S-phase kinase Cdc7-Dbf4	ND	Apoptosis, role in DNA-damaged cells when the DNA is nicked, replication of DNA
H4	Serine 1	Yeast, mammals	CK II	ND	DNA damage repair, mitosis and chromatin assembly
	Histidines 18 and 75	Mammals	Unknown	ND	Facilitation of DNA replication by destabilizing histone octamer

[a] DSB, double-strand break; ND, not determined.

Dephosphorylation in turn is the removal of a phosphate group by hydrolysis, it is assured by phosphatases. For example, fallowing DNA damage repair, a chromatin-associated phosphatase, Wip1, dephosphorylates H2A.X at S139 and allowing the cell to pass from the cell cycle checkpoint (Macurek et al., 2010).

Ubiquitination and Sumoylation

Ubiquitin is a highly conserved eukaryotic protein of 8,6kDa. Ubiquitination is the addition of one or more ubiquitin molecules on the ε-amino group of lysine residues. This reaction is catalyzed by the combined activity of three enzymes: E1-activating, E2-conjugating and E3-conjugating enzymes (Hershko et al., 1998). In the cytoplasm, ubiquitinated proteins are sent to the proteasome for degradation. Within chromatin, ubiquitination of histones does not lead to degradation. For example, mono-ubiquitination of histone H2B on K119 is associated with activation and elongation of transcription (Vitaliano-Prunier et al., 2008; Pavri et al., 2006) while the mono-ubiquitination of H2A on K120 or 123 is implicated in maintaining transcriptional repression by the polycomb repressive complex (Wang et al., 2004). Ubiquitination is a reversible event and polyubiquitin chains can be removed by specific isopeptidases called deubiquitinases (Nijman et al., 2005).

Sumoylation is a similar process to ubiquitination where small ubiquitin like modifiers (SUMO) of ~12kDa are attached reversibly to lysine residues in a reaction catalyzed again by E1, E2 and E3 enzymes. Sumoylated lysines of histones have been reported to be subsequently ubiquitinated, acetylated or methylated, suggesting a cross-talk between sumoylation and other post-translational modifications (Hendriks et al., 2014).

Other Modifications

There is an increasing number of histone modifications being described and studied, such as ADP-ribosylation, deamination, propionylation, butyrylation, citrullination and crotonylation. These research show that histones are intensely modified both on their amino tails and their core domains (Tan et al., 2011).

1.2.5. Histone Variants

Histone variants are nonallelic isoforms of conventional histones and are present in all eukaryotic organisms. There are variants for all classes of histones, except for H4. Unlike canonical histones, the expression of histone variants is not restricted to the S phase but may occur throughout the cell cycle. Furthermore the expression of certain subtypes can even be restricted to a specific tissues or specific stages of development.

Genes encoding the histone variants are present only in one or two copies and are distributed throughout the genome. They all have at least one intron and a poly-adenylation signal.

The differences between histones and their variants can affect the overall stability of the nucleosome or the residues of the nucleosome that are exposed. Hence the incorporation of histone variants confer distinct structural and functional properties, and thus distinct roles, to the nucleosome (Marzluff et al., 2002; Kamakaka and Biggins, 2005).

1.1.1.1.H2A Family Variants

The H2A family includes the largest number of variants, including the H2A.Z and H2A.X variants, found in most eukaryotes, and H2A.Bbd and macroH2A variants, specific to vertebrates.

Histone variant H2A.Z

H2A.Z represents approximately 5-10% of total histone H2A and is highly conserved through evolution derived from a single origin, (Malik et al., 2003) sharing ~60% sequence homology with the canonical H2A. Its removal is lethal in Drosophila (Daal and Elgin, 1992) and mouse (Faast et al., 2001). Nucleosomes containing variant H2A.Z instead of histone H2A are less stable at the interaction between the H2A.Z/H2B dimer and $(H3/H4)_2$ tetramer, hence a less stable nucleosome, which may allow easier access to the double helix of DNA (Abbott et al., 2001; Suto et al., 2000).

H2A.Z is involved in transcriptional activation and preventing heterochromatin formation (Meneghini et al., 2003). H2A.Z is enriched at eukaryotic promoters, specifically at the +1 and −1 nucleosomes, flanking the nucleosome free regions associated with RNA pol II-transcribed genes and facilitating transcription (Raisner et al., 2005; Zhang et al., 2005; Weber et al., 2014).

Posttranslational modifications of H2A.Z are also deterrent of its functions. For example mono-ubiquitination of H2A.Z is associated with facultative heterochromatin and X-inactivation while acetylation correlates with active genes (Sarcinella et al., 2007; Millar et al., 2006; Bruce et al., 2005). Genome wide deposition of H2A.Z is diverse, dynamic and regulated, in mouse trophoblast stem cells H2A.Z is lost from gene promoters and locates to the centromeres during mitosis (Nekrasov et al., 2012). H2A.Z in fission yeast is excluded from centromeric chromatin (Ogiyama et al., 2013). Interestingly,

even though its role may differ in these organisms, knockdown of H2A.Z leads to defects in chromosome segregation in both mice and fission yeast (Rangasamy et al., 2004; Kim et al., 2009).

Histone Variant H2A.X

H2A.X amounts up to a quarter of the total amount of histone H2A in the cell and possesses a conserved C-terminal tail motif containing a phosphorylatable serine (S139). The phosphorylation occurs in response to DNA double-strand breaks, where foci, termed γ-H2A.X, appear within ten minutes after cells are exposed to ionizing radiation that introduces double strand breaks and disappear gradually within two hours (Rogakou et al., 1998). H2A.X in its phosphorylated form, γ-H2A.X, is believed to destabilize chromatin and impair H1 binding (Downs et al., 2000). It has been called the guardian of the genome as it recruits and triggers DNA repair mechanisms and induces chromatin remodeling near the break site (Thiriet and Hayes, 2005). However H2A.X is dispensable to the repair process since DNA repair was observed in H2AX-deficient cells and animals signaling the existence of independent repair pathways (Yuan et al., 2010).

Histone Variant MacroH2A

MacroH2A is atypical histone variant, although its amino-terminal domain shares ~60% similarity with canonical H2A, macroH2A is characterized by a large C-terminal extension that can measure up to 200 amino acids in length according to species, called the macrodomain. Nucleosomes containing macroH2A are less flexible and more hydrophobic (Chakravarthy et al., 2005), their presence in a nucleosome disturbs both the recruitment of transcription factors such as NF-kB, nucleosome remodeling by the SWI / SNF complex (Angelov et al., 2003) and that histone acetylation necessary for transcription (Doyen et al., 2006b). Hence macroH2A is associated with the suppression of transcription and is particularly enriched on the inactive X chromosome (Mietton et al., 2009).

Phylogenic distribution of macroH2A is not uniform and knock-out of this variant in mice isn't lethal, however knock-out mice do exhibit impaired pre- and postnatal growth in addition to male reproductive impairments (Talbert et al., 2012; Pehrson et al., 2014).

Histone Variant H2A.B

The histone variant H2A.B, also known as H2A.Bbd is found, contrary to macroH2A, to be excluded from the inactive X chromosome (Bbd for Barr-deficient body) and is

associated with transcriptionally active chromatin (Chadwick and Willard, 2001). H2A.B shares only ~50 % sequence similarity with canonical H2A and has a truncated docking domain. H2A.B containing nucleosomes bind only ~120 bp of DNA and are less stable than conventional nucleosomes (Bao et al., 2004; Doyen et al., 2006a; Gautier et al., 2004). H2A.B nucleosomes also lack the small acidic region that forms on the surface of the canonical nucleosomes as well as K119 whose ubiquitination is linked to transcriptionally inactive regions (Zhou et al., 2007). More recently, H2A.B has also been found to localize at DNA replication and repair sites in elongating spermatids (Arimura et al., 2013).

1.1.1.2.H2B Family Variants

Contrary to the large diversity of H2A variants, few H2B variants have yet been identified. The most studied variants are the mammalian TH2B (Testis specific H2B) and H2B.FWT. TH2B shares 86% similarity with the canonical H2A and largely replaces conventional H2B in spermatocytes in humans, mouse and rats (Churikov et al., 2004). In humans, it is expressed throughout spermatogenesis, where it is found in at the telomeres (Zalensky et al., 2002). In mice, TH2B directs the final stages of histone to protamine transition in male germ cells and reassembles back after fertilization on the male genome during protamine to histone exchange (Montellier et al., 2013).

H2B.FWT is a surprisingly divergent variant and shares only 45% sequence identity with conventional H2B, sequence divergence is most pronounced in the amino-tail on account of the 42 amino acid extension of the H2B.FWT tail. The reconstitution of nucleosomes in vitro containing this variant showed H2B.FWT does not disturb the nucleosome remodeling by SWI / SNF, but its amino tail prevents H2B.FWT contribution during mitotic chromosome assembly. H2B.FWT is also associated with telomeric regions (Boulard et al., 2006).

1.1.1.3.H3 Family Variants

The H3 family includes two conventional subtypes H3.1 and H3.2, and three variant subtypes H3.3, H3.4 (a.k.a H3.t or TH3) and CenH3 (a.k.a CENP-A). With the exception of CenH3, all subtypes diverge from one another by only few residues. For instance H3.1 and H3.2 differ only by one residue.

Histone Variant H3.3

The histone variant H3.3 diverges from H3.1 by only five residues and from H3.2 by four residues. Nucleosomes containing these H3 subtypes are highly similar in structure. Intriguingly, H3.3 specific residues are highly conserved and are located on the accessible surfaces of the H3/H4 tetramer, including several PTM sites (Melters et al., 2015).

In mammals, H3.3 is coded by two genes H3F3A, H3F3B and disruption of either one of theses genes causes detrimental effects on development (Couldrey et al., 1999; Bush et al., 2013; Szenker et al., 2011). H3.3 has been associated with transcriptionally active chromatin (Ahmad and Henikoff, 2002; McKittrick et al, 2004) and is supposed to be a hallmark of active genes (Hake and Allis, 2006), although this role has recently been challenged given the presence of H3.3 in constitutive heterochromatin regions such as telomeres and pericentric chromatin (Szenker et al., 2011; Goldberg et al., 2010).

As mentioned before H3S10 and H3S28 phosphorylation are important for chromosome condensation at the beginning of mitosis. They first appear in prophase, persist until anaphase, and localize to peripheral regions of the condensed DNA. Strikingly, H3.3 is phosphorylated on S31 (H3.3 S31P) during mitosis but differs from H3S10P and H3S28P, in both timing and location, as it is present only in late prometaphase and metaphase (Hake et al., 2005).

Histone Variant H3.4

The variant H3.4, diverges from H3.1 by four residues and H3.2 by five residues, it has been identified as a testis specific variant. In rats, it represents up to 40% of total histone H3 throughout spermatogenesis (Meistrich et al., 1985). In humans, its expression is restricted to spermatocytes, where it potentially represents the majority histone H3 (Witt et al., 1996). H3.4 nucleosomes appear less stable than conventional nucleosomes, including a weaker interaction between the H3.4:H4 tetramer and H2A:H2B dimers. H3.4 has been also reported in other tissues, such as the brain and the embryo (Tachiwana et al., 2010).

Histone Variant CenH3

The variant CenH3 (CENP-A) is the centromere specific histone variant and is considered to be the epigenetic marker of centromeric chromatin. It is the central protein in this thesis work and will be thoroughly described in the final chapter of the introduction.

2. Mitosis

In eukaryotes the cell cycle of proliferating cells can be divided into two parts: interphase and mitosis. Mitosis is the part of the cell cycle where a eukaryotic cell equally divides its duplicated genetic material, resulting in two cells with the same genome. However interphase isn't a stagnate state, it encapsulates specific stages during which both cell growth and DNA replication occur in preparation for cell division. Hence the cell cycle can be examined in four discrete phases. The M phase of the cycle corresponds to mitosis, which is usually followed by cytokinesis. The next phase is the G(ap)1 phase, which corresponds to the interval "gap" between mitosis and DNA replication where the cell is metabolically active and grows continuously. G1 is followed by the S(ynthesis) phase corresponding to DNA replication. The S phase is followed by the G2 phase, during which cell growth and protein synthesis continues in preparation for mitosis (Cooper GM (2000). "Chapter 14: The Eukaryotic Cell Cycle". *The cell: a molecular approach*.).

2.1. Stages of Mitosis

Mitosis is divided into five phases and starts with prophase (Figure 2.1a), during which the interphase chromatin condenses to form individualized chromosomes and transcription is widely paused (Kadauke et al, 2013). Animal cells contain a pair of centrosomes responsible for microtubule nucleation and anchoring. During prophase each centrosome migrates to the poles of the cell, forming a bridge of spindle fibers (Eddé et al., B.1990).

The next stage is prometaphase (Figure 2.1b), the nuclear membrane disintegrates and the chromosomes that are now fully condensed migrate towards the cells equatorial plane. This movement is called congression. Kinetochore microtubules emerging from the centrosomes attach to the kinetochores and spindle microtubules move the chromosomes toward the center of the cell. The chromosomes align on the metaphase plate in metaphase (Figure 2.1c), during which the cell ensures the correct orientation and bipolar attachment of each chromosome to the mitotic spindle.

Metaphase is then followed by anaphase (Figure 2.1d), where the sister chromatid cohesion is broken and each chromatid pulled towards the opposing poles of the cell.

In telophase (Figure 2.1e) the migration of chromatids is completed and the nuclear envelope reforms while microtubules of the mitotic spindle dissociate and chromatin decondensation occurs. Alongside telophase, the division of the cytoplasm called cytokinesis takes place (Figure 2.1f). A contractile ring forms and tightens gradually and

leads to the near total separation of the two daughter cells. The disappearance remaining small dense structure, called the residual body marks the end of cell division.

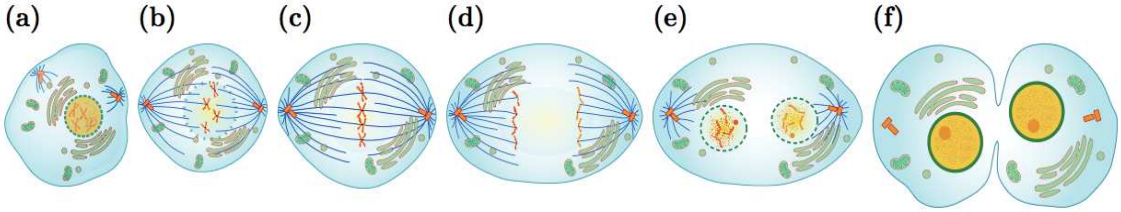


Figure 2.1 Stages of mitosis. **a)** Prophase: chromosomes condense and the nuclear membrane disappears. **b)** Prometaphase: chromosome capture by microtubules. **c)** Metaphase: Alignment of chromosomes on the metaphase plate. **d)** Anaphase: sister chromatids separate and migrate towards the poles. **e)** Telophase: chromosomes decondensation and nuclear membrane reformation. **f)** Cytokinesis: division of the cytoplasm. (by Marina Ruiz Villarreal)

2.2. Regulation of Mitosis

Ensuring proper progression of mitosis is exceptionally important as errors during cell division can have devastating consequences for the cell. Transcriptional arrest during mitosis mandates that all effectors and enzymes are readily available in the cell, thus activation of molecular pathways depend on protein turnover and posttranslational modifications. Two mechanisms ensure this control. The first is removal of effectors by proteolysis when their function is no longer needed, and the second is phosphorylation that serves as molecular on and off switch for many enzymes. Hence mitotic kinases play a central role in controlling mitosis.

Principle Kinases

This section only covers the role of these kinases during mitosis and does not envelope functions during the rest of the cell cycle.

CDK Family Kinases

Cyclin Dependent Kinases (CDKs) are among the most important conductors of the cell cycle. During mitosis, the predominant kinase family is Cdk1, of the twenty Cdks that have been identified in humans, Cdk1 is the only one that is essential for the cell cycle in all eukaryotic cells (Malumbres et al., 2009). Expressed during G2 phase, Cdk1 is phosphorylated on Tyr15 by Wee1 kinase and becomes inactive (Parker et al., 1992). Cdc25 phosphatase dephosphorylates Cdk1 and Cdk1 association with cyclin B allows the

activation of the kinase and triggers mitotic entry (Ferguson et al., 2005; Nilsson and Hoffmann, 2000).

The cyclinB/Cdk1 complex targets multiple proteins. The phosphorylation of lamins causes them to dissociate and depolymerize resulting in disintegration of the nuclear envelope. The phosphorylation of condensins induces chromosome condensation (Heald et al., 1990). Other targets include microtubule and kinesin-related proteins, promoting the assembly of the mitotic spindle and cohesins, located on chromosome arms (Kimura et al., 1998). Finally, during the transition from metaphase to anaphase, degradation of cyclin B initiated by the ubiquitin ligase anaphase promoting complex/cyclosome (APC/C), opens the way for completion of mitosis (Murray, 2004).

PLK family kinases

The polo-like kinases (PLK) are conserved serine/threonine kinases involved in the regulation of cell cycle progression through G2 and mitosis. Expressed during G2 phase, they are initially distributed throughout the cell but later localize from prophase to metaphase to the centrosomes and kinetochores. (Glover et al., 1998)

PLK1 mediates mitotic entry, both by direct activation of the cyclin B/Cdk1 complex and by phosphorylation of Cdc25c, which promotes its nuclear localization (Roshak et al., 2000). At the centrosomes, it promotes the recruitment of γ-tubulin by phosphorylating Asp, enabling centrosome maturation and microtubule nucleation (Petronczki et al., 2008). On the kinetochore, it contributes to recruitment of NDC80 complex involved in bipolar spindle formation (Wong and Fang, 2005). Finally, PLK1 participates in the metaphase to anaphase transition, either by directly phosphorylating the APC/C subunits (Golan et al., 2002) or by promoting the degradation of one of its inhibitors, Emi1.

NRK family kinases

NIMA (never in mitosis A) kinases have been identified in *Aspergillus nidulans* in a screen for cell cycle mutants. NIMA mutants remained arrested in G2 phase without ever entering into mitosis and acquired the name never in mitosis A. NIMA homologues are NRK family or NIMA-related kinases (O'Connell et al., 2003).

In vertebrates, the most likely functional homologue is the NEK2 kinase, essentially important in the maturation and separation of centrosomes and bipolar spindle formation. NEK2 controls centrosome separation (essential for the formation of bipolar spindles) by

phosphorylating proteins, such as Nap1, resulting in their displacement from the centrosomes (Bahe et al., 2005). It also regulates kinetochore microtubule attachment stability in mitosis via phosphorylation of NDC80 (Chen et al., 2002).

Aurora family kinases

Part of the serine/threonine kinases, Aurora kinase was first identified in Drosophila in a screening for defective chromosome segregation. Although in yeast one gene encodes for Aurora kinase, the family includes three members in mammals, Aurora A, Aurora B, and C. Aurora C has more limited expression and plays an important role during meiosis (Tang et al., 2006) and early embryonic development (Fernandez-Miranda et al., 2011; Schindler et al., 2012). Aurora A and B are involved in somatic mitosis and share some common characteristics: they are both expressed in G2 and M phases and their activation depends on their association with specific cofactors coupled to auto-phosphorylation. Lastly, they are both degraded at end of mitosis by APC/C dependent ubiquitination (Lindon et al., 2016).

Aurora A mainly localizes at centrosomes and mitotic spindle poles throughout mitosis, assisting in centrosome maturation and separation, microtubule dynamics, and bipolar spindle assembly (Barr and Gergely, 2007). In contrast, Aurora B localizes at centromeres and chromosomes in early mitosis and locates to the spindle midzone/midbody during mitotic exit (Figure 2.2) (Adams et al., 2001; Schumacher et al., 1998).

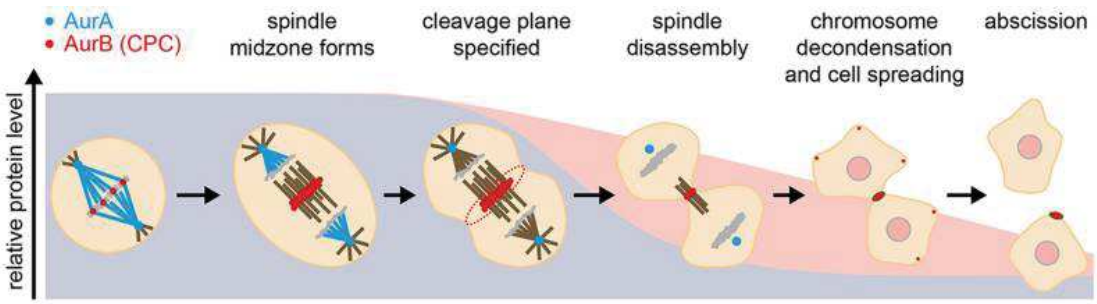


Figure 2.2 Aurora kinase dynamics throughout mitosis (adopted from Lindon et al., 2016)

Aurora A

Aurora A contributes to mitotic entry and maturation of centrosomes. It initially displays a cytoplasmic localization, where it forms a complex with its cofactor Bora. The complex Aurora A/Bora in turn phosphorylates PLK1 (Macůrek et al., 2008; Seki et al.,

2008), which promotes mitotic entry (by PLK1 action on the complex cyclin B / Cdk1) and the phosphorylation and degradation of Bora. Aurora A then associates with another cofactor TPX2 (Chan et al., 2008) and the complex can locate to the centrosome (Figure 2.2). There, it allows the recruitment of pericentriolar material (PCM) (Hannak et al., 2001) and the mobilization of microtubule-associated proteins (Dutertre et al., 2002). It also phosphorylates the centrosomic fraction of Cdc25, maintaining the local activation of the cyclinB/Cdk1 (Dutertre et al., 2004).

More recent studies suggest also Aurora A involvement in cytokinesis by insuring the stability of astral microtubules and central spindle robustness (Lioutas and Vernos 2013; Reboutier et al., 2013).

Aurora B

Aurora B is a member of chromosomal passenger complex (CPC), a set of proteins characterized by their location profile during mitosis. This complex, in particular the protein INCENP (inner centromere protein), allows both the complete auto-activation of the kinase but also its proper localization. Aurora B associates with chromosome arms in prophase, where it participates in chromatin condensation by phosphorylating condensins and histone H3 at serine 10 (Giet and Glover, 2001; Lipp et al., 2007). It also assists in cohesin removal along chromosome arms, together with PLK1 (Giménez-Abián et al., 2004). During prometaphase and metaphase, Aurora B localizes to centromeres and oversees the attachment of microtubules to kinetochores by destabilizing incorrect attachments (Shang et al., 2003; Liu and Lampson, 2009; Tanaka et al., 2002; Lampson et al., 2004; Cimini et al., 2006; Knowlton et al., 2006; Lampson and Cheeseman, 2011) and activating the spindle assembly checkpoint (SAC). During telophase, Aurora B is located on the spindle midzone for required disassembly (Buvelot et al., 2003). Lastly during cytokinesis, it is located at the residual body where it contributes to the formation of the contractile ring (Figure 2.2) (Minoshima et al., 2003).

More recently a control mechanism involving Aurora B activity gradient has been proposed as a regulator of late mitosis. Complete separation of chromosomes and their arrival to the spindle poles is necessary for DNA decondensation and complete nuclear envelope reformation (NER). At the spindle midzone as chromosomes migrate to opposite poles, the lack of accessible substrates to become dephosphorylated by phosphatases ultimately drives mitotic exit. In this regard Aurora B phosphorylation gradient at the

spindle midzone would allow for the spatial regulation of several substrates involved in chromosome (de)condensation and NER, such as histone H3 (Hsu et al., 2000; Neurohr et al., 2011). DNA decondensation and NER are inhibited on lagging chromosomes/ chromatin bridges as a chance for possible reintegration (Afonso et al., 2014; Karg et al., 2015). This indicates a potential surveillance mechanism or "chromosome separation checkpoint" that controls the extent of anaphase chromosome separation before completion of telophase (Maiato et al., 2015).

2.3. The mitotic spindle

2.3.1. Microtubules

The mitotic spindle is composed of microtubules, a cylindrical structure formed by the association of thirteen protofilaments each composed of α- and β-tubulin heterodimers (Nogales, 2001). Microtubules are polarized structures, the plus end only exposes α-tubulin and the minus end only exposes β- tubulin molecules. The protofilaments are of parallel orientation so the overall structure also maintains polarity.

Microtubules are highly dynamic structures, their length varies by constant addition or removal of tubulin dimers at the polymer ends and microtubule polymerization alternates between slow and rapid depolymerization phases according to the local concentration of tubulin dimers (Desai and Mitchison, 1997). At a given end, the authorization of polymerization rate and frequency of depolymerization determine whether the microtubule is in growth or shrinkage. Each end may exhibit different dynamic properties, which gives the microtubule the opportunity to grow or shrink in a particular direction. When the dynamic properties of the two ends are such that one end is growing as the opposite end decreases as the tubulin dimers seem to "treadmill" over the microtubules to the other end, microtubule length remains constant (Margolis and Wilson, 1998).

2.3.2. Centrosomes

The centrosome is the major microtubule organizing center of the cell. It is duplicated during S phase and the resulting two centrosomes define the poles of the cell and hence that of the mitotic spindle during mitosis (Urbani and Stearns, 1999). Centrosomes consist of a pair of centrioles (themselves an assembly of nine microtubule triplets) surrounded by pericentriolar material (PCM).

A major component of PCM is the γ-tubulin ring complex (γ-TuRC) that nucleates microtubules (Zheng et al., 1995). During mitosis the nucleation capacity of the centrosome is increased by the recruitment of larger amounts of γ-TuRC (Khodjakov and Rieder, 1999, Piehl et al., 2004). This allows a rapid increase in the number of microtubules issued from the centrosome and thus the formation of the future mitotic spindle. However, the centrosome is not strictly necessary in this process, and a functional mitotic spindle can form in the absence of centrosomes (Mahoney et al., 2006). The centrosome also has an important role in cell cycle regulation (Doxsey et al., 2005).

2.3.3. The kinetochore

The kinetochore is a protein complex transiently associated with centromeres during mitosis. Spindle microtubules attach to chromosomes through kinetochores.

When observed by electron microscopy, the kinetochore has a structure revealing three layers: the inner kinetochore which is very closely associated with chromatin, the outer kinetochore, a thick region of 50 to 60 nm that interfaces with microtubules and central kinetochore, an electron sparse region between the inner and outer layers. A network of fibers, the fibrous ring is associated with the external surface of the kinetochore (McEwen et al., 2007).

The simplest kinetochore is that of *Sacharromyces cerevisiae*, it consists of about sixty proteins and contains only a single anchoring site for microtubule attachment (McAinsh et al., 2003; Westermann et al., 2007). Out of five of the main complexes in *S. cerevisiae* (CBF3, Ndc80, Mtw1, Spc105 and Dam) three of them, Ndc80, Mtw1 (called MIS12 in humans) and Spc105 (Knl1 humans) are conserved in higher eukaryotes (Musacchio and Salmon, 2007; Cheeseman and Desai, 2008; Welburn and Cheeseman, 2008). Kinetochores of higher eukaryotes are bulkier and each bind numerous microtubules (15 to 20 microtubules for the human kinetochore) and are formed of a repetition of the elementary module found in yeast (Joglekar et al., 2008). The constitutive centromere associated network (CCAN) is also conserved in all eukaryotes and is supposed to be the foundation on which the rest of the kinetochore proteins are assembled. Contrary to the other members of the kinetochore those of the CCAN are permanently associated to the centromeres and are not transitional like the rest of the kinetochore.

The elementary functional module kinetochore is the Knl1-MIS12-Ndc80 network (KMN), a 10-subunit supercomplex consisting of the three conserved complexes Ndc80,

Mtw1/MIS12 and Spc105/Knl1 (Cheeseman et al., 2006). In this network, the complex Ndc80 directly interacts with microtubules, with increasing affinity when combined with Mtw1/MIS12 and Spc105/Knl1 complexes (Figure 2.3). The 4-subunit Ndc80 complex forms an elongated structure with two globular heads at each end, separated by a long super-coiled region (Ciferri et al., 2007). One end of the cylinder, composed of globular regions Ndc80 and Nuf2, directly binds microtubules (Cheeseman et al., 2006; Wei et al., 2007); the other end composed of globular regions Spc24 and SPC25 (Wei et al., 2006), is associated with the inner kinetochore (Figure 2.3b). Each microtubule is bound to the kinetochore by several KMN complexes (Joglekar et al., 2006).

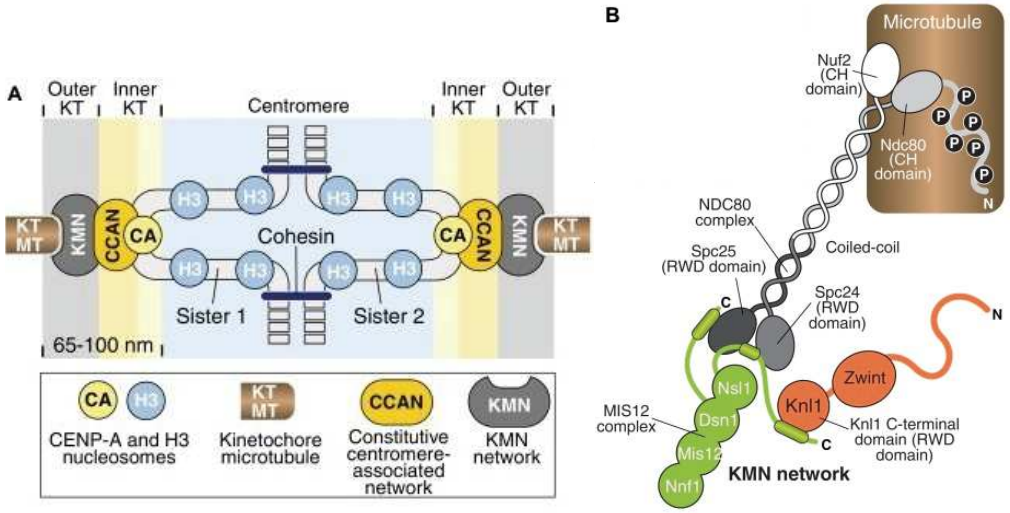


Figure 2.3 a) Schematic representation of the centromere **b)** Schematic view of the main interactions of the KMN network (adopted from Pesenti et al., 2016)

The Mis12 complex interacts directly with the inner kinetochore via CENP-C (part of the CCAN). The interactions with the Ndc80 complex and with the 2-subunit Knl1 complex take place on adjacent binding sites at the opposite end of the Mis12 complex. They involve short linear motifs of the Nsl1 and Dsn1 subunits of the Mis12 complex, which engage RWD (RING finger, WD repeat, DEAD-like helicases) domains of the Ndc80 complex and the Knl1 complex (Pesenti et al., 2016).

2.4. Chromosome dynamics during mitosis

2.4.1. Microtubule Attachment

All chromosome movement during mitosis requires attachment to the mitotic spindle. Microtubules that attach to the kinetochore form K-fibers, these microtubules are oriented with their plus ends to the kinetochore and their minus ends to the centrosome.

Two models postulate how chromosomes are attached to microtubules of the mitotic spindle during prometaphase, though they are not mutually exclusive. The main model "search and capture" (McIntosh et al., 2002) suggests that microtubules emitted from centrosomes explore space in all directions until they contact a kinetochore. Microtubules that have established contact stabilize while the rest is depolymerized. This mechanism is enabled in particular by increasing the nucleation of microtubules at the centrosomes, the increased recruitment of γ-tubulin and the action of Aurora A (Khodjakov and Rieder, 1999; Brittle and Ohkura, 2005). The search and capture mechanism is not relevant to spindle assembly in cells lacking centrosomes, which include higher plants and some animal oocytes but surprisingly studies have shown that animal cells in the absence of centrosomes can still form functional spindles (Hinchcliffe et al., 2001).

The model of "self-assembly" proposes that microtubules are formed in the immediate vicinity of chromatin, independent of centrosomes. A part of them at least are suspended by the kinetochores and oriented parallel to form a K-fiber (McKim and Hawley, 1995). This model could explain the formation of a mitotic spindle function observed in the absence of centrosomes (Mahoney et al., 2006).

2.4.2. Congression

Congression is the process by which, as the name indicates, chromosomes congregate to the metaphase plate in prometaphase. One of the major forces responsible for these movements comes from the depolymerization of positive ends of microtubules associated with the kinetochore when the shortening of the resulting K-fiber causes chromosomes to be pulled towards the corresponding pole. On the opposite kinetochore, there is polymerization of positive ends of microtubules and elongation of the K-fiber, but this phenomenon is not strong enough to "push" the chromosome to the metaphase plate (Khodjakov and Rieder, 1996). So the movement towards the metaphase plate is not direct, chromosomes oscillate back and forth until polymerization rate stabilizes as microtubules on opposite sides reach equal length and chromosomes are aligned at the metaphase plate.

Another mechanism contributing to the congression is the lateral displacement of chromosomes along an already formed K-fiber. The sliding of chromosomes by laterally attached kinetochores is mediated by the minus-end-directed motor dynein (Yang et al., 2007) or the plus-end-directed kinesin CENP-E (Kapoor et al., 2006; Kim et al., 2010) along with polar ejection forces (PEFs) exerted mainly by the chromokinesin Kid.

Chromokinesins are microtubule plus-end-directed motor proteins present on the chromosome arms harboring both chromatin- and microtubule-binding domains. As a consequence of their motor activities, chromokinesins move chromosomes away from the poles by generating random polar ejection forces (PEFs). Lateral movements allow congression of the not yet bioriented chromosome and promote bi-orientation by mobilizing the chromosome to the metaphase plate and therefore to the microtubules emitted by the opposite pole (Iemura and Tanaka, 2015).

2.4.3. Segregation

Segregation of chromatids to spindle poles and hence to future daughter cells during anaphase is the result of two separate movements called anaphase A and B. During anaphase A chromatids migrate towards the poles facing them. During anaphase B the poles themselves migrate in opposite directions. The contribution of each movement to segregation of chromatids varies between cell types and species and their timing are not mutually exclusive.

Anaphase A

The movement of the chromatids toward the poles during anaphase A is the result of two main mechanisms: microtubule depolymerization and the activity of motor proteins in the kinetochore.

The depolimerization of microtubules at their positive end at the kinetochore causes the shortening of K-fibers and the traction pulls the chromatids towards the poles (Gorbsky et al. 1987; Gorbsky et al., 1988). The same effect can also be obtained by depolymerization to the negative end (on the side of the centrosome) coupled with polymerization of the positive end, granted that the rate of depolymerization surpasses the rate of polymerization (Khodjakov and Kapoor, 2005; Kwok and Kapoor, 2007).

Several motor proteins appear to be involved in the movement of chromosomes towards the poles, although the exact role of each of them remains unclear. One of the first identified is dynein, found associated with kinetochores during anaphase and exhibiting motor activity directed towards the negative end (Pfarr et al., 1990; Steuer et al., 1990) suggested to deliver proteins promoting depolymerization at the poles (Gaetz and Kapoor, 2004). Other proteins include members of the kinesin-13 family that catalyse microtubule depolymerisation by bending tubulins at microtubule ends (Desai et al., 1997; Ganem and Compton, 2004).

Anaphase B

The central spindle forms between segregating chromosomes by microtubules that have not attached to kinetochores but have continued to expend form the central spindle; an array of antiparallel microtubules from opposite sides that are bundled at their overlapping plus ends. Molecular motors are recruited to zone between the overlapping ends and generate forces that push the opposing ends of the central spindle further apart. This "*expension*" of the central spindle contributes to the movements of the centrosomes and further separates the poles (Brust-Mascher and Scholey, 2002; Brust-Mascher et al., 2004; Zhu et al., 2005). In addition, the central spindle is required for the final step of cytokinesis, cell separation or abscission (Glotzer et al., 2009). Astral microtubules that are not part of the mitotic spindle and link centrosomes to the cell cortex, also contribute to the migration of centrosomes (Rosenblatt, 2005).

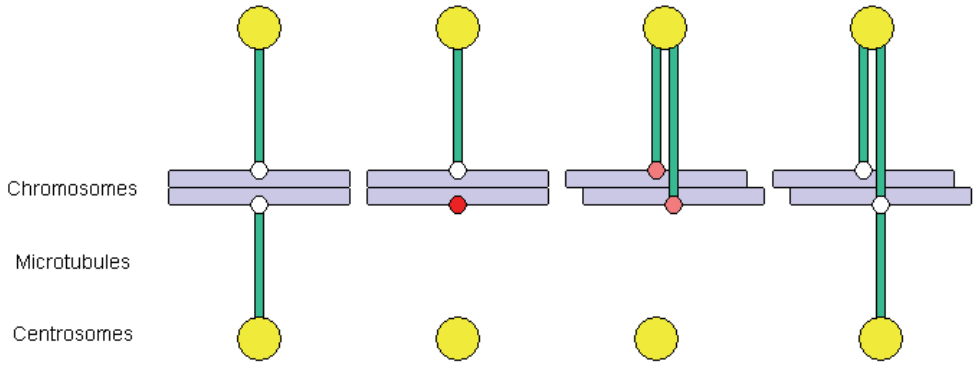
2.4.4. Spindle Assembly Checkpoint

The main goal of mitosis is to accurately separate sister chromatids, when mitosis is observed by live-microscopy the cell appears to briefly halt movement during metaphase. This halt is quite misleading since there is considerable molecular activity during this time. The spindle assembly checkpoint (SAC) is an important control mechanism to prevent the cell from entering anaphase precociously before chromosomes are properly attached to the mitotic spindle. Proper attachment is amphitelic attachment (Figure 2.4); the kinetochore of each chromatid is attached to K-fibers from the centrosome facing it. Other possible types of attachments are: monotelic attachment, where only on of the two kinetochores is attached; syntelic attachment, where the both kinetochores are linked to the same centrosome; and merotelic attachment, where one kinetochore is linked to two centrosomes (Figure 2.4). In the presence of unattached or incorrectly attached chromosomes, SAC delays the transition from metaphase to anaphase until the attachments are corrected (Maiato et al., 2004).

Metaphase to Anaphase Transition

The major effector of the metaphase to anaphase transition is the APC/C-Cdc20 complex. It has an E3 ubiquitin ligase activity directed especially against securin, a stoichiometric inhibitor of separase. Once securin is degraded, separase is no longer inhibited and can degrade SMC3, a protein member of the cohesin complex that holds together sister chromatids (Haering and Nasmyth, 2003). The destruction of SMC3 allows

separation of sister chromatids and entry into anaphase. Cohesin connects the sister chromatids along their entire length, from S phase until mitosis. In yeast, all cohesin is maintained until the metaphase to anaphase transition. However, in animal cells the vast bulk of cohesin rings along chromosome arms is removed from DNA in prophase and prometaphase by WapI and centromeric cohesin is protected. This results in the separation of chromosome arms, but not centromeres resulting in the classical X shape of mitotic chromosomes (Haarhuis et al., 2014). Another target of the APC/C-Cdc20 is cyclin B1, whose degradation signals the end of the activity of the cyclin B1-Cdk1 complex and promotes mitotic exit.



Configuration	Amphitelic	Monotelic	Syntelic	Merotelic
Centromeric tension	Yes	No	No	Yes
Spindle checkpoint	Inactive	Active	Active	Inactive

Figure 2.4 Different anchoring configurations between chromosomes and the mitotic spindle (adopted from Maiato et al, 2004).

SAC Activation

The presence of an incorrectly attached kinetochore activates the mitotic spindle checkpoint (Figure 2.4). SAC is inactivated once all kinetochores are properly attached (Rieder et al., 1994).

SAC activation prevents APC/C ubiquitin ligase from recognizing cyclin B and securin by catalysing the incorporation of the APC/C co-activator, CDC20, into the Mitotic Checkpoint Complex (MCC). MCC is composed of proteins Mad2, BubR1, BUB3 and Cdc20 where Mad2 and BubR1 inhibit Cdc20 by binding to substrate and APC/C recognition motifs (Chao et al., 2012; Han et al., 2013). In the absence of attachment to microtubules, the kinetochore acts as a catalytic site that promotes the formation of MCC (Howell et al., 2004; Shah et al., 2004) in particular by allowing the recruitment Mad2 and

conformation change, allowing its association with Cdc20 (Musacchio and Salmon, 2007). Once the kinetochores are attached, the components of the checkpoint are evacuated by motor proteins, ending MCC production and thus lifting the APC/C inhibition.

Detection of Incorrect Attachments

Monotelic and syntelic attachments (Figure 2.4) result in loss of spindle tension between the two kinetochores of the same chromosome (Kelly and Funabiki, 2009). The lack of kinetochores being stretched outwards from opposite sides trigger SAC activation. Merotelic attachments generate kinetochore tension, however they don't always activate the spindle checkpoint (Maresca et al., 2010). In the absence of sufficient tension, the Aurora B kinase that is localized at the centromeres during metaphase destabilizes the interactions between the kinetochore and microtubules. Aurora B may act by directly phosphorylating members KMN network, reducing their affinity for microtubules (Welburn et al., 2010). It can also phosphorylate mitotic centromere-associated kinesin (MCAK), challenging its microtubule activity (Knowlton et al., 2006). The overall effect of Aurora B activity is the breaking of incorrect attachments; it does not activate SAC directly. The proposed model for Aurora B activity depends on its distance from its substrates at the outer kinetochore, the force exerted on bi-oriented kinetochores separates the Aurora B gradient at the inner centromere from its outer kinetochore substrates, making Aurora B less able to access these substrates. Though CPC has been found enriched at merotelic attachments, these kinetochores are deformed and stretched compared to bi-oriented kinetochores, perhaps sufficient or homogenous extension can't take place and thus substrate availability for Aurora B is compromised and SAC is not always activated (Lampson and Cheeseman, 2011).

3. Centromeric Chromatin

The centromere is a highly differentiated chromosomal structure that governs the assembly of the kinetochore and allows the equal distribution of the genetic material. There are essentially two types of centromeres: those of monocentric chromosomes, which contain a single regionally localized centromere that generally appears as a constriction on the mitotic chromosome; and those of holocentric chromosomes where the centromere is distributed over the entire length of the chromosome. Most eukaryotes have monocentric chromosomes with the exception of some nematodes, insects and plants. The rest of this chapter will focus on monocentric chromosomes.

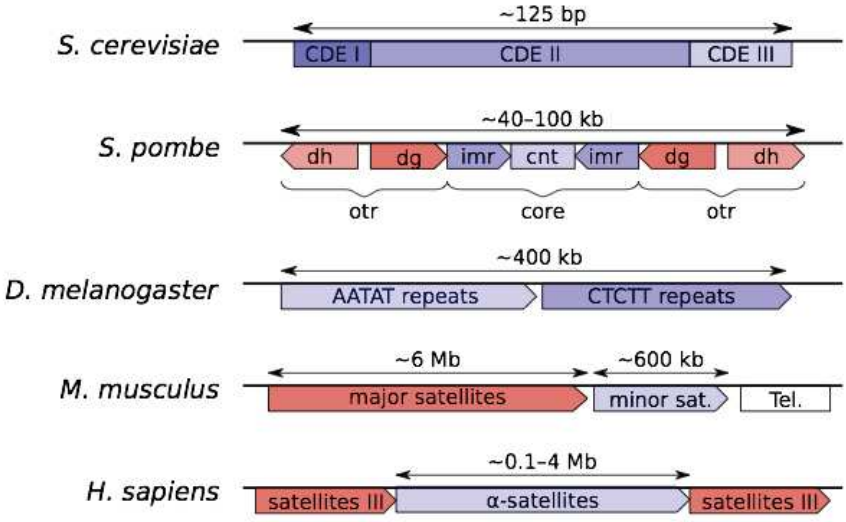


Figure 3.1 Centromere organization in different eukaryotes (blue for centromeric and red for pericentromeric chromatin). CDE, *Centromere DNA element*; cnt, *central core*; imr, *innermost repeats* ; otr, *outer repeats* ; Tel, telomeric DNA. (by Damien Goutte-Gattat)

3.1. Centromere Determination

Centromeric DNA is highly divergent between species in terms of length as well as sequence conservation (Sullivan et al., 2001). Centromeres may be point centromeres, found in some budding yeast including *S. cerevisiae* that contain short DNA sequences or regional centromeres that contain kilo- to megabases of DNA (Pluta et al., 1995). *S. cerevisiae* has the simplest centromeres that include a 125bp sequence containing three conserved functional elements CDEI, II and III (Figure 3.1). *S. pombe* centromeres consist of regions of 40 to 100 kb containing a single central sequence of 4 to 5 kb flanked by repeat sequences. In Drosophila, the only centromere characterized at the DNA sequence corresponds to a 420kb region, containing tandem repeats of satellite DNA interrupted by transposable elements. In *Mus musculus* the centromere core is composed of minor

satellite arrays containing homogenous 120 bp repeats flanked by less ordered ~234 bp major satellite repeats. In humans, the centromeres span multiple megabases and consist of 170 bp repetitions of α-satellite DNA (Figure 3.1).

The absence of homology between centromeric sequences and the high diversity of satellite repeats amongst species suggest that centromere identity can't simply depend on DNA. Multiple studies show that centromeric DNA is neither necessary nor sufficient to support the function of the centromere with the exception of point centromeres. Functional centromeres can form on non-repetitive euchromatic areas offering no similarity with centromeric regions (Marshall et al., 2008) and the mere presence of centromeric type DNA doesn't lead to a functional centromere (Karpen and Allshire 1997). In fact dicentric chromosomes that have acquired a second centromere by chromosome fusion will functionally inactivate one of the two centromeres (Earnshaw et al., 1985; Sato et al., 2012). Hence centromere identity depends on chromatin properties rather than sequence and is defined epigenetically. Unlike the DNA sequence, the chromatin associated with centromeres has relatively well conserved features in eukaryotes.

3.2. Centromeric Chromatin Structure

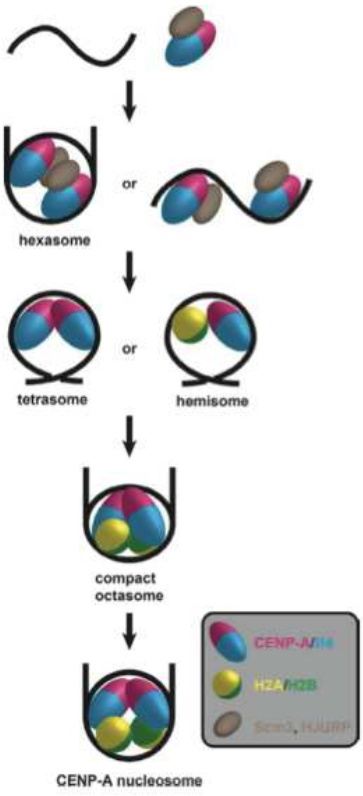


Figure 3.2 Possible models for CENP-A nucleosome formation.

Centromeric chromatin was initially considered to be heterochromatin, however centromeric chromatin is a unique and specialized type of chromatin distinct from heterochromatin as well as from euchromatin. Centromeric chromatin in all eukaryotes is characterized by the presence of the histone variant CENP-A (CenH3), which is believed to be the epigenetic mark responsible for determining centromere identity.

Before the resolution of the CENP-A nucleosome structure in 2011, several models had been proposed for CENP-A nucleosomes (Figure 3.2). The octosome model, where the CENP-A nucleosome is on octamer resembling H3 nucleosomes, the hemisome model where the nucleosome is a tetramer consisting of a single copy of the four histones H2A, H2B, CENP -A and H4 (Furuyama and Henikoff, 2009) and the compact nucleosome model where

the interface between two CENP-A molecules is rotated compared to the H3-H3 leading to an overall more compact nucleosome structure (Sekulic et al, 2010). Additionally. the hexasome model in yeast, where the nucleosome is composed of two copies of histones H4 and CENP-A, and two copies of the CENP-A chaperone HJURP (Mizuguchi et al., 2007) and finally, the tetrasome model consisting of two dimeric CENP-A-H4s (Williams et al., 2009).

After the resolution of CENP-A nucleosome structure, models other than the octosome model has been proposed to represent intermediate stages of maturation CENP-A nucleosomes.

CENP-A nucleosomes are octosomes that are not compact (Tachiwana et al., 2011a) and have a structure globally similar to that of H3 nucleosomes. However, unlike the canonical H3 nucleosome, only the central 121 base pairs of the nucleosomal DNA are tightly bound to the histone octamer, instead of 147 base pairs in the H3 nucleosome. This indicats that thirteen base pairs at each end of the nucleosomal DNA are disordered, furthermore this region contains the CENP-B box where the centromeric protein B (CENP-B) binds (Figure 3.3c). The CENP-B box is the only known centromere sequence conserved between primates and rodents.

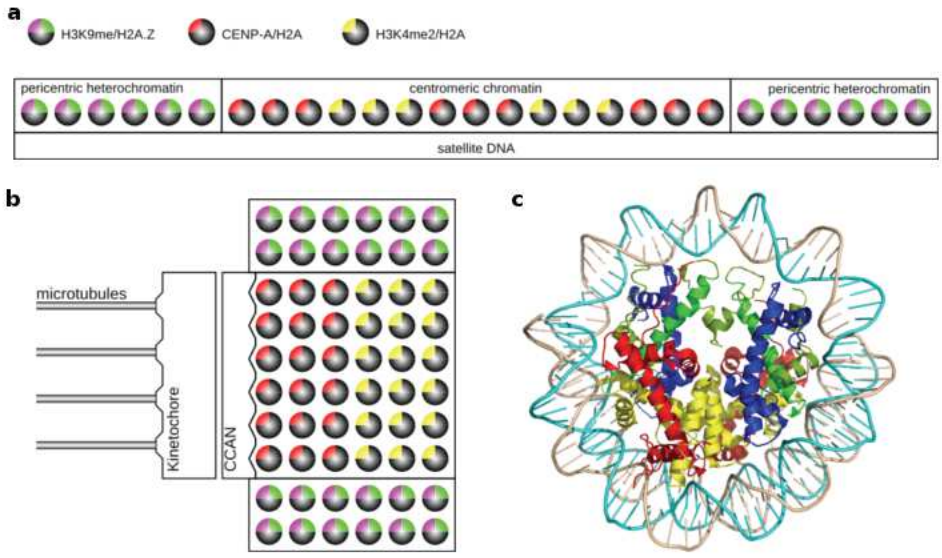


Figure 3.3 Structure of centromeric chromatin. **a)** Linear layout of centromeric and pericentromeric chromatin. **b)** Spatial organization of centromeric chromatin upon condensation of chromosomes in mitosis. (by Damien Goutte-Gattat) **c)** Crystal structure of the centromeric CENP-A nucleosome as resolved by Tachiwana et al. (2011).

Centromeric chromatin organization consists of alternating regions containing CENP-A nucleosomes and regions containing conventional nucleosomes (Figure 3.2a) and each of

these regions extend over about 15 to 40kb (Blower et al., 2002). During condensation necessary to achieve the mitotic chromosome, these regions of the nucleofilament form a higher order structure so that all the CENP-A nucleosomes are exposed at the surface of centromere whereas conventional nucleosomes are placed at the inner centromere (Figure 3.2b). Additionally, H3 nucleosomes that are a part of centromeric chromatin are dimethylated on lysine 4 of H3 (H3K4me2) but are also hypoacetylated (Sullivan and Karpen, 2004).

The region of chromatin located on either side of the centromeric chromatin containing CENP-A nucleosomes is the pericentromeric chromatin. It differs epigenetically from centromeric chromatin and shows typical marks of heterochromatin, including the methylation of H3 lysine 9 associated with the presence of the protein HP1 (Sullivan and Karpen, 2004). In addition, pericentromeric chromatin is enriched in histone variant H2A.Z (Figure 3.3a, b)(Greaves et al., 2007). Furthermore H2A.Z is targeted to the centromeres before cells enter mitosis in a transcription independent manner, indicating a role in centromere organization (Nekrasov et al., 2012; Boyarchuk et al., 2014).

3.3. Centromeric Chromatin and Transcription

It has long been assumed that centromeric chromatin was heterochromatin and thus was not transcribed. However, more and more centromeric trascripts are being identified suggesting that the transcription of DNA underlying to the centromeric chromatin is possible. Centromeric transcripts have already been detected in budding yeast, Drosophila, mammals and plants (Rosic et al., 2014; Ohkuni et al., 2011; Bouzinba-Segard et al., 2006; Neumann et al., 2007), encoding genes that can reside and be expressed in centromeric regions (Yan et al., 2006; Saffery et al., 2003). Transcription from centromeric chromatin influences CENP- A loading in yeast, flies and a 1.3 kb long α-satellite transcript co-immunoprecipitates the soluble CENP-A pre-assembly complex that contains the CENP-A chaperon HJURP in humans. Furthermore, the knock-down of α-satellite RNA leads to altered Aurora B activity and an increase of H3S10 phosphorylation. Mounting evidence is showing that centromeric transcription and transcripts are emerging players in centromere regulation (Rošić et al., 2016).

3.4. The Centromeric Histone Variant CENP-A

3.4.1. Structure and Conservation

Centromeric protein A (CENP-A) is a histone variant of the H3 family. It exists in all eukaryotes under various names; Cse4p in *S. cerevisiae*, CNP1 in *S. pombe*, CID in *Drosophila melanogaster* and CENP-A refers to the mammalian homologues. The name CenH3 (Centromeric H3) has been proposed to designate this protein uniformly regardless of species.

CENP-A shares the overall structure of canonical core histones and is composed of two distinct domains, a carboxy-terminal globular domain and an unstructured amino-terminal domain or tail. The globular domain of CENP-A is conserved amongst species and has 62% of sequence similarity with the corresponding domain of histone H3 and has a major role in the incorporation of CENP-A into the nucleosome. This is assured by a specific domain called the *CENP-A Targeting Domain* (CATD), that is composed of the second α-helice and the L1 loop of CENP-A (Black et al., 2008). The CATD also allows tighter binding of CENP-A to H4, conferring a conformational rigidity to the nucleosome. The CENP-A specific histone chaperone HJURP has been shown to interact with this domain and regulate centromeric chromatin assembly (Shuaib et al., 2010). As opposed to the globular domain, the amino-terminal domain of CENP-A is highly divergent and unstructured and will be discussed in more detail in a later section. This distinction between the two domains is also reflected upon comparing different CenH3 proteins between species (Figure 3.4).

The importance of the globular domain of CENP-A appeared very early, as it is needed for specific centromeric localization of the protein (Sullivan et al., 1994). The essential part of the functions of CENP-A has subsequently been reduced to a shorter domain, the CENP-A targeting domain (CATD) consisting only of the L1 loop and α2 helix of histone-fold domain (Figure 3.5a) (Black et al., 2004). Crystallographic data highlights in particular the importance of two residues located in the L1 loop that make the CENP-A L1 loop longer than that of H3 and for this reason the CENP-A L1 loop protrudes out from the surface of the nucleosome (Tachiwana et al., 2011a).

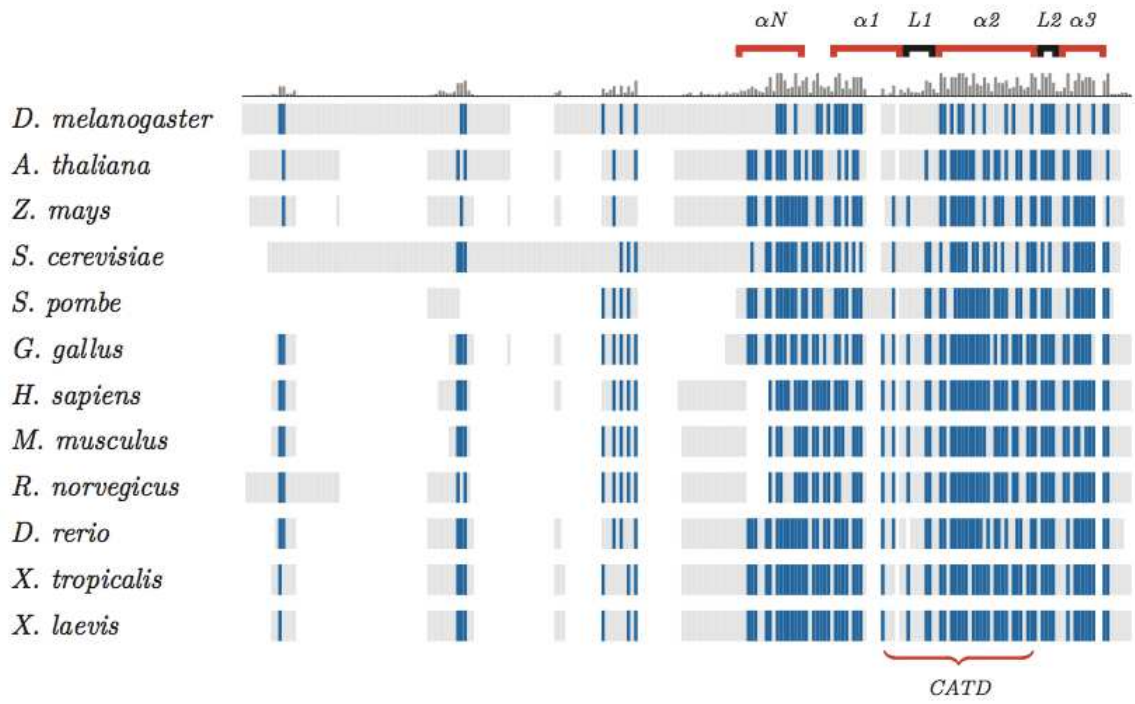


Figure 3.4 Alignment of CenH3 sequences in multiple species.

The CATD contains the binding site for CENP-A chaperone HJURP (Foltz et al., 2009; Shuaib et al., 2010). The CATD also confers the CENP-A/H4 tetramer a more rigid conformation then the H3/H4 tetramer (Black et al., 2007a), allowing the targeting CENP-A to centromeric chromatin. Additionally the α-N helix of CENP-A is implicated in conferring flexibility to the DNA ends of the CENP-A nucleosome, when substituted with the α-N helix of H3, the nucleosome rigidifies and enables H1 binding (Roulland et al., 2016). The carboxy-terminal tail of CENP-A makes extensive contacts with CENP-C the key component of the CCAN.

3.4.2. Maintenance of CENP-A at the Centromeres

DNA is replicated during each cycle hence the overall histone load would be diluted by half if new histones were not loaded onto the nascent DNA. For conventional histones, deposition is S phase dependent (Osley, 1991) so this is not the case. However CENP-A deposition is not dependent on the S phase and CENP-A is not expressed during this phase (Shelby et al., 1997). Since the presence of CENP-A in centromeric chromatin is required for centromere function and maintenance, the correct deposition of new CENP-A into centromeric chromatin is crucial. Thus it is no surprise that CENP-A deposition is a complex process with many steps that requires a compulsory passage through mitosis in

metazoans (Jansen et al., 2007).

During replication of the centromeric DNA, CENP-A nucleosomes are distributed evenly between the two nascent DNA molecules, how the gaps are filled is still not well understood. It has been thought that gaps that remain are either left empty or more likely occupied by conventional nucleosomes containing histone H3. A 2011 study showed that H3.3 nucleosomes fill the gaps left by CENP-A during S phase and act as a placeholder until G2 phase or until mitotic exit, till the new CENP-A is deposited (Figure 3.5b) (Dunleavy et al., 2011). During G2 phase, CENP-A starts to be expressed and newly synthesized CENP-A proteins are taken in charge by the specific chaperone, HJURP however HJURP doesn't locate to the centromeres till early G1 (Shuaib et al., 2010; Dunleavy et al., 2009; Foltz et al., 2009).

In addition to HJURP, CENP-A deposition in G1 requires the three subunit MIS18 complex, composed of MIS18α, MIS18β and MIS18-binding protein1 (M18BP1). Intriguingly, in Drosophila, the functions of the MIS18 complex and HJURP are combined into a single molecule, Chromosome alignment defect 1 (Cal1) (Chen et al., 2014).

M18BP1 interacts with CENP-C and since CENP-C binds directly to CENP-A nucleosomes, this ensures that the MIS18 complex and HJURP target preexisting centromeres for the incorporation of CENP-A (Dambacher et al, 2012; McKinley et al., 2014). Once CENP-A is incorporated at centromeres, CENP-C, the RSF (remodelling and spacing factor) complex and MgcRacGAP (male germ cell Rac GTPase-activating protein) assure centromeric maintenance (Figure 3.5b) (Falk et al., 2015; Perpelescu et al., 2009; Lagana et al., 2010).

The presence of transcriptionally permissive marks and the absence of heterochromatin in the centromere are also important for CENP-A deposition. Chromatin remodelers associated with active transcription including RSF1, FACT (facilitates chromatin transcription), chromodomain helicase DNA-binding protein1 (CHD1), retinoblastoma-binding protein p46 (RBAP46) and RBAP48 facilitate new CENP-A deposition by assuring the necessary transcriptionally permissive centromere core or by directly participating in remodelling centromeric chromatin (McKinley and Cheeseman, 2016).

The finely tuned deposition of CENP-A relies heavily on phosphorylation by CDKs (Figure 3.5c). For instance in Drosophila, cyclin A degradation is crucial for CENP-A

deposition (Mellone et al, 2011). In human cells, M18BP1 phosphorylation by CDKs reduce its centromeric localization (Silva et al., 2012) and prevents MIS18α and MIS18β recruitment outside G1 (McKinley et al., 2014). Additionally, HJURP localizes to the centromeres outside of G1 phase when CDKs are inhibited or if HJURP is non phosphorylatable (Muller et al., 2014).

In addition to temporal regulation by CDKs, CENP-A deposition is regulated locally and requires a licensing step by PLK1 (Figure 3.5c). PLK1 is required for CENP-A deposition downstream of CENP-C localization but upstream of MIS18α recruitment. It binds to and phosphorylates the MIS18 complex to promote MIS18 complex localization to license CENP-A deposition at the centromere. Thus, CENP-A deposition is regulated by a two-step mechanism; CDKs prevent CENP-A deposition outside of the G1 phase by inhibiting MIS18 complex localization and assembly as well as HJURP recruitment and PLK1 binds to the MIS18 complex to promote CENP-A deposition at centromeres during G1 (McKinaly et al., 2014).

Contrary to metazoans, in *S. pombe* CNP1 deposition occurs during the S and G2 phases (Takayama et al., 2008). The chaperone SIM3 binds newly synthesized CNP1 and delivers it to a second chaperone Sim1 (counterpart HJURP and SCM3). The direct interaction between Mis16-Mis18 complex (counterparts RbAp46 / 48 and Mis18α / β) and Sim1 is necessary for CNP1 deposition (Mellone et al., 2009; Williams et al., 2009).

3.4.1. CENP-A and Kinetochore Assembly

Though kinetochore function is highly conserved, kinetochore composition is more diverse as kinetochore proteins seem to have undergone recurrent evolutionary turn over. Even amongst lineages that have similar kinetochore composition, individual function and essentiality of proteins can be different (Drinnenberg et al., 2016). Centromeric chromatin containing CENP-A is the platform on which kinetochore proteins assemble, in the absence of CENP-A there is no kinetochore formation (Blower and Karpen, 2001). CENP-A overexpression results in ectopic CENP-A localization that in turn leads to incorrect localization of at least part of the kinetochore proteins (Van Hooser et al., 2001). CENP-A is at least partially responsible for recruiting the centromeric CCAN complex, which represents the interface between centromeric chromatin and the inner kinetochore (Cheeseman and Desai, 2008).

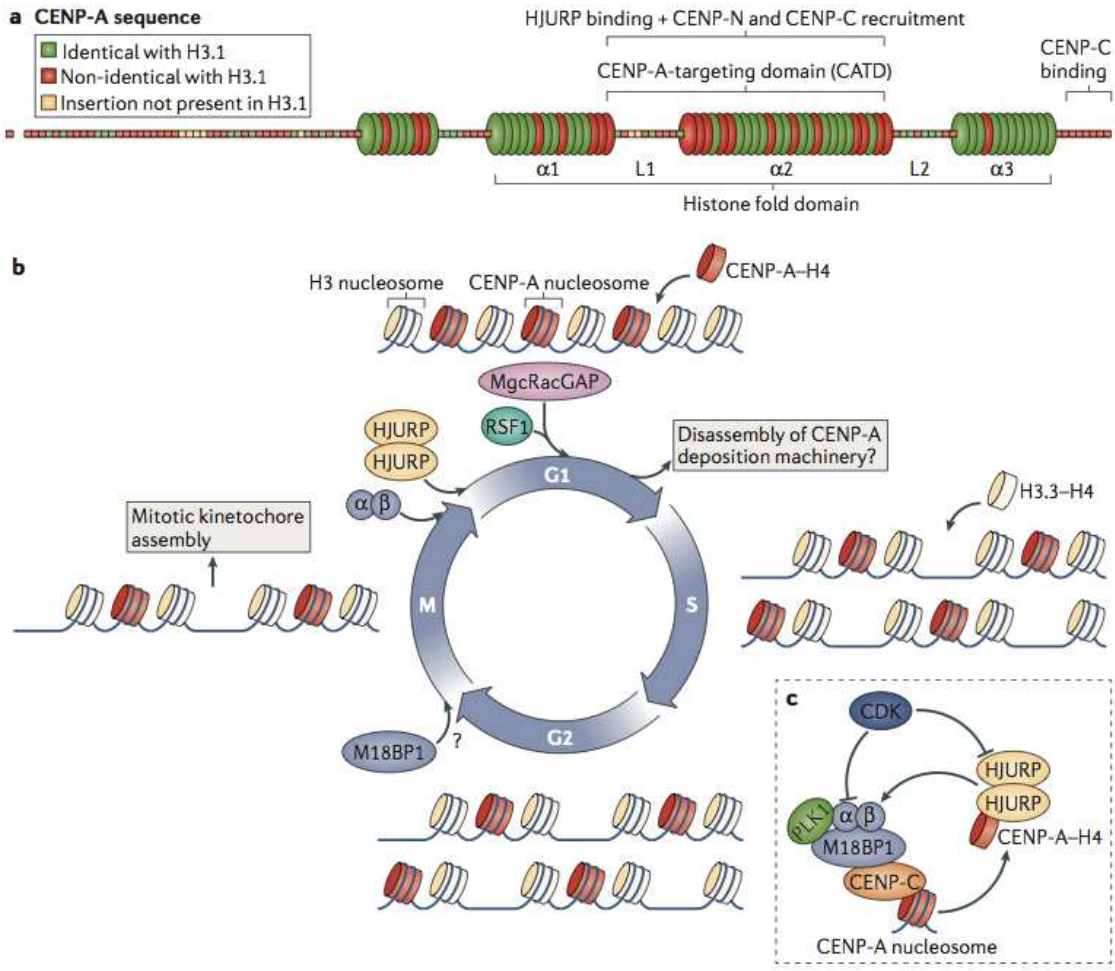


Figure 3.5 Specialization and propagation of CENP- A **a)** Human CENP-A primary and secondary structure, showing conservation with histone H3 **b)** Changes in CENP-A chromatin during the cell cycle **c)** Model for the two-step regulation of CENP-A deposition (Adopted from McKinley et al, 2016)

In humans, the CCAN can be subdivided into two sub-complexes according to their relationship with CENP-A nucleosomes. The first is the CENP-A nucleosome-associated complex (CENP-A^{NAC}), composed of CENP-C, CENP-H, CENP-M, CENP-N, CENP-T and CENP-U that interacts directly with CENP-A nucleosomes. The second is the CENP-A distal complex (DAC) composed of CENP-K, CENP-L, CENP-O, CENP-P, CENP-Q, CENP-R and CENP-S that interact with members of CENP- A^{NAC} (Foltz et al., 2006).

The CCAN proteins can also be combined into five sub-complexes: CENP-C, the CENP-L-N complex, the CENP-H-I-K-M complex, the CENP-O-P-Q-U-R complex and the CENP-T-W-S-X complex (McKinley et al., 2016).

CENP-A nucleosomes are recognized by CENP-C and CENP-N (Carroll et al., 2010). The two proteins recognize different elements of the CENP-A nucleosome. CENP-C specifically recognizes a short C-terminal peptide (LEEGLG) of CENP-A, which is

enough to locate CENP-C on the centromere, while CENP-N recognizes the CATD (Carroll et al., 2009). CENP-C and CENP-N are likely to associate simultaneously and independently with the CENP-A nucleosome, where they can recruit other members of the CCAN (Carroll et al., 2010). Several CCAN proteins bind directly to DNA, including CENP-C (Sugimoto et al., 1994) and the CENP-T-W-S-X complex. Members of the CENP-T-W-S-X complex contain histone-fold domains and assemble a structure similar to that of canonical nucleosomes that can wrap DNA, protecting ~100 bp region from nuclease digestion (Nishino et al., 2012). However DNA binding is not sufficient for the centromeric localization of the CENP-T-W-S-X complex and also requires contact with the CCAN (Nishino et al., 2012; McKinley et al., 2015).

Two parallel but non-redundant pathways are assured by CENP-C and CENP-T for the recruitment of the KMN network (Nishino et al., 2013; Przewloka et al., 2011). The forced incorporation of CENP-C and CENP-T is sufficient to assemble a functional ectopic kinetochore, regardless of CENP-A presence (Gascoigne et al., 2011a). CCAN interactions are regulated so to only assemble full kinetochore during mitosis. For instance, phosphorylation by AuroraB kinase promotes interactions between CENP-C and the MIS12 complex during mitosis (Gascoigne and Cheeseman, 2013).

In addition to their roles in kinetochore assembly, as it was mentioned in the previous chapter, CCAN proteins play a role in resisting the forces generated by spindle microtubules as well as regulating CENP-A deposition. It is worthy to underline the key importance of CENP-C in the CCAN and the kinetochore as it interacts directly with the CENP-A nucleosome as well as with centromeric chromatin. The proper localization of CENP-C is essential for the assembly of other CCAN proteins and it links the centromeres with the microtubule binding interface of kinetochores. CENP-C is also important for correct CENP-A deposition (Klare et al., 2015; Dambacher et al., 2012; McKinley and Cheeseman, 2016; Falk et al., 2015).

3.4.2. Other Functions of CENP-A

There have been reports about CENP-A function outside of its cell cycle dependent centromeric function. In senescent cells CENP-A levels in centromeric chromatin are drastically reduced (Maehara et al., 2010). This reduction is accompanied by centromeric chromatin acquiring a heterochromatin state resulting in a progressive loss of centromeric function. Furthermore, suppression of CENP-A expression is capable of inducing senescence in a p53-dependent manner. The level of CENP-A was also found diminishing

with age in human pancreatic cells as it becomes almost undetectable after 30 years (Lee et al., 2010). These observations suggest a role for CENP-A in aging.

It has also been reported that CENP-A is recruited to the DNA double-stranded breaks (Zeitlin et al., 2009), proposing possible involvement of CENP-A in DNA repair. In Drosophila, CHRAC14 an essential component of the *Drosophila* DNA damage response can interact with CID (CENP-A) and prevent inappropriate CID incorporation at sites of damaged chromatin (Mathew et al., 2014).

3.4.3. CENP-A and Pathology

Chromosomal aneuploidy and genome instability are common hallmarks of cancer. CENP-As key role in kinetochore assembly suggests direct involvement of CENP-A in the development of certain tumors. Indeed, CENP-A is overexpressed and found on non-centromeric regions in colorectal and breast cancers as well as in hepatocellular carcinoma and lung adenocarcinoma (Vardabasso et al., 2014; Tomonaga et al., 2003). Overexpression of CENP-A and segregation defects that result could be a mechanism by which the loss of the retinoblastoma protein creates a favorable genomic instability in tumor development (Amato et al., 2009). A study of testicular germ cell tumors identified CENP-A as one of the main markers of this type of cancer, with an increased expression by a factor of 20 compared to healthy testicular tissue (Biermann et al., 2007). Recent translational work has shown that CENP-A is elevated in tumor cells compared to normal, in particular in primary human colorectal cancer tissues (Tomonaga et al., 2003). Increased expression of CENP-A is associated with higher-grade cancers and increased invasiveness (Ma et al., 2003). Consequently, CENP-A has been included in predictive genetic profiles in breast cancer (Sotiriou et al., 2006). These observations suggest that elevated CENP-A may be correlated with poorer patient outcomes. CENP-A may also provide unique prognostic and predictive information in estrogen receptor (ER)-positive breast cancer (McGovern et al., 2012).

CENP-A is also involved in autoimmune pathologies as the centromere is targeted by autoantibodies. In fact the first human centromeric proteins were identified with autoantibodies from patients with CREST syndrome, a form of systemic sclerosis (Palmer et al., 1987). These autoantibodies could disrupt the proper functioning of the centromere, explaining aneuploidy observed in these pathologies (Jabs et al., 1993).

3.4.4. The Amino-terminal Domain of CENP-A

CENP-A differs from histone H3 mainly by its NH_2-terminus. Though the globular domain of CENP-A is very important in centromeric function, several reports also point to the amino-terminal domain, however the main function of this domain remains unclear. NH_2-terminal tail is present in all species and is highly divergent, both in length and sequence while the globular domain is conserved (Figure 3.4) and has received little attention. Several studies, however, suggest that this domain can play an important role.

In *S. cerevisiae*, an essential function has been attributed to a part of the NH_2-terminal domain of Cse4p, called the essential NH_2-terminal domain (END). This domain is necessary for chromosome segregation and assembly (Chen et al., 2000), moreover its absence is lethal (Morey et al., 2004). In Drosophila, an arginine-rich motif in the NH_2-terminal domain of CID is involved in recruiting BubR1, a key protein of the mitotic spindle checkpoint (Torras-Llort et al., 2010) and this pattern appears relatively preserved in CID counterparts. In humans, CENP-A overexpression and the resulting misincorporation of CENP-A at non-centromeric regions, assembles a subset of centromere-kinetochore components, including CENP-C, hSMC1, and HZwint-1 by a mechanism that requires the CENP-A amino-terminal tail. (Van Hooser et al, 2001).

More recent work has proposed diverse functions for the amino-tail using rescue experiments with chimeric protein mutants. In human diploid cells it was shown that kinetochore assembly requires along with the CATD domain of CENP-A either the amino- or the carboxy-terminal tail by stabilizing CENP-B binding to human centromeres or direct recruitment of CENP-C, respectively. The same study found that in fission yeast the CATD and the amino-terminal domain were both necessary for centromere function (Fachinetti et al., 2013). Additionally, Cnp1 amino-terminus mutants were shown to be viable but exhibited elevated levels of chromosome loss and centromere inactivation. Amino-terminus mutants had reduced levels of CENP-T branch of the CCAN but not that of CENP-C (Folco et al., 2015).

A separate study in U2OS cells revealed that in early stages of centromere establishment both tails of CENP-A are required along with the CATD domain. Additionally, amino-tail plays a CENP-B independent role in centromere establishment and indirectly stabilizes CENP-C binding, thereby directly or indirectly promoting CENP-T recruitment (Logsdon et al., 2015).

The constraints on the sequence of the N-terminal domain seem low, which is consistent with the absence of conservation. In humans, CENP-A is phosphorylated on serine 7 during prophase and prometaphase (Zeitlin et al., 2001b). Phosphorylation is transient and is no longer detectable from telophase on. It appears to be mediated by Aurora A in prophase and Aurora B in prometaphase (Kunitoku et al., 2003). It is necessary for proper chromosome congression and segregation as well as for the completion of cytokinesis (Zeitlin et al., 2001a). A separate study in HeLa cells showed that the mitotic phosphorylation of human CENP-A is required for kinetochore function (Goutte-Gattat et al., 2013) as the absence of amino-tail phosphorylation resulted in an increase chromosome segregation defects and partial loss of CENP-C from the centromeres. The same study showed that the mitotic phosphorylation of human CENP-A is required for kinetochore function. The absence of amino-tail phosphorylation resulted in partial loss of CENP-C from the centromeres. Direct interactions between the phosphorylated form of CENP-A and 14-3-3 proteins as well as between 14-3-3 proteins and CENP-C were also demonstrated which suggests that the indirect interaction with the amino-tail that stabilizes CENP-C can be through the 14-3-3 proteins. The serine located at position 7 in the primary sequence of CENP-A that is phosphorylated is not conserved in other species. In addition, no phosphorylation of the amino-terminal tail of CENP-A from other metazoan was reported. This raises the question whether the reported data on the mitotic function of human CENP-A phosphorylation can be generalized or if it is restricted to human cells only (Goutte-Gattat et al, 2013).

4. Objectives

The function of the amino-terminal domain of CENP-A and its interactions remains unclear. The purpose of this thesis will be to investigate the role of this domain during mitosis. In this study we would like to further our understanding by addressing the following questions:

Is the N-terminal domain of CENP-A necessary for successful mitosis?

As mentioned in the last chapter of the introduction, there has been multiple studies that implicate the NH_2-terminal domain of CENP-A in mitosis and genome stability. However there is conflicting opinion about its overall importance and its function. We would like to unambiguously tie its function to mitosis by removing the CENP-A tail only during mitosis but no techniques are currently available to achieve this. During this thesis work, we have devised a new method that we call the "Hara-kiri" approach in order to answer the above question in human HeLa cells.

Is the phosphorylation of CENP-A involved in its mitotic function?

It has been shown that the amino-terminal tail of CENP-A is phosphorylated in mitosis at S7 in human cells and that this phosphorylation is important for successful mitosis but S7 is not conserved in other species. In addition, no phosphorylation of the amino-terminal tail of CENP-A from other metazoan was reported. This raises the question whether the reported data on the mitotic function of human CENP-A phosphorylation can be generalized or whether it is restricted only to human cells. Mouse CENP-A amino-tail has multiple serine residues, we aim to assess the role of phosphorylation of CENP -A amino-tail in mouse.

PART II

5. Materials and Methods

5.1. Plasmid constructions

PCR amplifications

All PCR amplifications were done in 50 μl of final volume using 100 ng of template DNA, 200 nM of each primer, 200 μM of dNTPs, 2.6 units of High Fidelity Taq polymerase (Roche™) and 5 μl of 10× PCR buffer. The program used is as follows: an initiation step at 94 °C (2 minutes) followed by 35 cycles consisting of a denaturation step at 94 °C (30 seconds), an annealing step at 55 °C (30 seconds) and an elongation step at 72 °C (1 minute/ kb); the program ends with a final elongation step of 72 °C (5 minutes). PCR products were then deposited on 1 % agarose gel and purified using the Wizard PCR & Gel Clean Up System (Promega™).

Vector constructions

Mouse CENP-A NH2-terminal domain constructs for MEF cells

The synthesis of the sequences of mouse CENP-A, H3-CENP-A, H3SA-CENP-A, SA-CENP-A and S15.16.22A-CENP-A was done by Eurofins. The construct H3-CENP-A was obtained by replacing the first 35 amino acids of the CENP-A amino tail with the first 42 amino acids of that of histone H3.1. H3SA-CENP-A is the same as H3-CENP-A construct except for S10 and S28 mutated to alanine. The construct SA-CENP-A has all 7 serine residues in the mouse CENP-A amino tail mutated into alanines while S15.16.22A-CENP-A has only the first three serine residues. These constructs were cloned directly into pCLMFG-HA-GFP vector previously modified from the #12341 Plasmid bought from Addgene.

Mouse RFP-CENP-C constructs for MEF cells

Mouse RFP-C2-CENP-C sequence was courtesy of the Cheeseman lab and was cloned into the pQCXIH vector purchased from Clonetech.

Hara-kiri constructs for HeLa cells

Three different constructs were used for this project. The first construct pBABE-GFP-Katana-nCENPA, expresses a fusion protein consisting of the GFP reporter gene, the Katana protease, and the silent-resistant CENP-A containing the Katana target site after residue 15. This vector was constructed via the PCR amplification of Katana-nCENPA sequence from a pre-existing pcDNA3.1-Katana-nCENPA vector in our lab. The reaction was carried out using the following primers: EcoRI-Katana, 5'-gaa ttc ATG GCG CCC

ATC ACG GC-3' and SalI-CENPA, 5'-gtc gac tta GCC CAG ACC TTC TTC CAG-3' (restriction sites and stop codon in lowercase). The PCR product was T/A-cloned into an intermediate pGEM-T Easy vector (Promega™), then into a pre-existing pBABE-GFP vector between EcoRI and SalI restriction sites.

The second construction was similar to the first but contained a Katana protease carrying a point mutation on Ser139. This mutation affects the catalytic triad of the protease and is thus supposed to produce an inactive, "dead" protease. Our lab was in possession of a plasmid in which site-directed mutagenesis had already been performed. From this plasmid we amplified the dead form of Katana that we will call "KatanaD". The PCR reaction was carried out using the following primers: HindIII-Katana, 5'-aag ctt ATG GCG CCC ATC ACG GCG TAC G-3' and BamHI-SPC-Katana, 5'-gga tcc cgt agc agg gac tct cga tgt cgc tgg aac acg aga TCT CAT GGT TGT CTC TAG G-3' (restriction sites and spacer sequence—to facilitate bending—in lowercase). The PCR product was T/A-cloned into a pGEM-T Easy intermediate vector and then subcloned into the previously mentioned pcDNA3.1-Katana-nCENPA so to replace the functional Katana. Once the pcDNA3.1-KatanaD-nCENPA vector was confirmed we transferred the KatanaD-nCENPA fusion to the pBABE-GFP vector using the same strategy as before. The third construct, pBABE-GFP-Katana-nnnCENPA contains two additional cleavage sites at the NH2-terminal domain of CENP-A and the sequence synthesis was done by Eurofins. Then the nnnCENP-A sequence was cloned into the pBABE-GFP-Katana-nCENPA so to replace the nCENP-A.

5.2. Cell lines

Cells were maintained in standard DMEM media containing 10% (vol/vol) FBS, 1% penicillin and streptomycin, and 1% glutamine at 37 °C in a 5% (vol/vol) CO_2 atmosphere. HeLa cell synchronizations were performed by double thymidine blocking using 2mM of thymidine (T9250 SIGMA). The first block was done for 18 hours fallowed by 9 hours release and 18 hours of second blocking. Cells released after the second block entered mitosis 8 hours later.

HeLa cell lines

HeLa cell lines stably expressing the Hara-kiri constructs were obtained by retroviral infection with murine leukemia virus (Moloney murine leukemia virus, MMLV) produced by amphotropic Phoenix packaging cells (Swift et al. Al., 2001). The ΦNX-ampho cells

were transfected into 10 cm dishes with 6 μg of DNA and 10 μl of Lipofectamine 2000 (Invitrogen) according to the suppliers' recommendations. The cells were then incubated at 37 ° C for two days.

The target HeLa cells were seeded at 30% confluence in 10 cm dishes. Two days after transfection of the ΦNX-ampho cells, their medium was collected and replaced with 10 ml of fresh medium. The viral supernatant was centrifuged for 5 minutes at 1200 rpm and then filtered through a 0.45 μm filter. For each target cell box, 5 ml of viral supernatant was mixed with 5 ml of fresh medium and 8 μg / ml of polybrene (Sigma Aldrich). The culture medium of the target cells was then replaced by this viral solution and the cells were replaced in the incubator. The infection was repeated three times in two days approximately every 12 hours. The day after the last infection, the target cells were seeded in new dishes and incubated in the presence of 3 μg / ml of puromycin.

CENP-A KI-cKO Mouse Lines

The CENP-A cKO/KI mouse line was established by using standard mouse genetic approaches. In this line, the CENP-A gene was replaced by a double tagged FLAG-HA-CENP-A fusion. The targeting vector of the generated cKO/ KI FLAG-HA tagged CENP-Aflox/flox mice flanked exon 1 of the murine CENP-A gene with LoxP recombination sites.

MEF Cell Lines

MEFs were isolated and cultured from day 14,5 embryos of the CENP-A cKO/KI mice and were immortalized via SV40 infection. Cells were cultivated in DMEM high glucose (4.5 g/l) and 10 % fetal bovine serum and 1% penicillin and streptomycin at 37 °C and 5 % CO2.

CRE recombinase expression for CENP-A knock-out was assured by MSCV CreERT2 (Addgene #22776) retroviral infection fallowed by 2μM (Z)-4-Hydroxytamoxifen (Sigma-Aldrich) treatment or by adenovirus infection (Ad-CMV-iCre, Vector Biolabs, Philadelphia, PA).

All cell lines resulting from infections with the GFP-CENP-A tail constructs were then FACSed (BD FACS Aria Cell Sorting System) to obtain cell lines with homogenous expression of the exogenous CENP-A at near endogenous expression level. MEFs infected with RFP-CENP-C constructs were subjected to clonal selection.

5.3. Immunocytochemistry and quantification of mitotic defects

Cells were fixed with 4% formalin solution (Sigma-Aldrich) for 20 minutes at 37 °C then washed 3 times with PBS. Cells were then permeabilized with 0.02 % Triton for 3 minutes. The coverslips were then successively incubated for an hour at 37 °C with primary anti-lamin (1:1000, Santa Cruz biotechnology) and secondary anti-goat Cy3 (1:300, SantaCruz) antibodies or with CREST anti-centromere auto-antibody (1:1000, Immunovision™ HCT0100) and the anti-human IgG coupled with Alexa594 (1:300, Invitrogen™ A11014) secondary antibody. Each incubation was followed by three 5-minute wash steps with PBS; DNA was stained in the presence of 1.5 µg of Hoechst 33342. The slides were later observed and imaged using a Zeiss Axiovision epifluorescence microscope with Plan-Apochromat 63x objective. GFP, cyanine-3, and Hoechst 33342 were used as fluorochromes. Images were acquired with a Zeiss Axiocam camera piloted with the Zeiss Axiovision software.

The rate of defects was calculated from a sample of 200 ± 25 mitotic cells for mitotic defects and 400 ± 25 nuclei for nuclear defects for MEF cells. The rate of defects for HeLa cells was calculated from a sample of 150 ± 25 cells per mitotic phase. Experiments were performed in triplicate and error bars indicate standard error of mean.

5.4. Nuclear isolations and immunoblotting

Cells were pelleted then resuspended in 250 µl of Buffer B (NaCl 15 mM, Tris HCl pH 7.5 15 mM, KCl 60 mM, sucrose 11 %, EDTA 2 mM, EGTA 0.5 mM, DTT 1 mM, spermine 0.2 mM, spermidine 0.65 mM, PMSF 0.5 mM). Next 250 µl of Lysis Buffer (Buffer B with 4 % NP-40) was added and the suspension was incubated 5 minutes on ice. The cell lysate was then successively centrifuged twice at 13,200 rpm for 5 minutes. After each centrifugation, the pellet was resuspended respectively in: 250 µl of Buffer D (Buffer B without EDTA and EGTA) and finally 150 µl of PBS with 1x Laemmli buffer. Samples where then sonicated at 200 J/W.s and used for immunoblotting. Samples were incubated at 100 °C for 10 minutes prior to electrophoresis on acrylamide gel in denaturing conditions (SDS-PAGE), then transferred onto a PVDF membrane.

The membrane was saturated using a blocking buffer (PBS 1×, 5 % milk, 0.3 % Tween) for 45 minutes, then successively incubated for 1 hour at room temperature with the primary, then with the secondary antibody. Incubations were followed by four 5-minute wash steps (once with PBS 1×, twice with PBS 1×, 0.4 M NaCl, 0.5 % Triton, and last

with PBS 1×, 0.4 M NaCl). Finally the membrane was prepared for revelation using the ECL Plus system (GE Healthcare™).

The primary antibody used for HeLa extracts was an anti-CENP-A (Epitomics™ 1745-1) diluted 500× in PBS 1×, 10 % FBS, 0.2 % Tween; the secondary antibody was an anti-rabbit IgG coupled to peroxydase (GE Healthcare™ 934V) diluted 5,000× in PBS 1×, 1 % milk, 0.02 % Tween. The primary antibody used for MEF extracts was an anti-HA (Abcam, ab9110) diluted 500× in PBS 1×, 10 % FBS, 0.2 % Tween; the secondary antibody was an anti-rabbit IgG coupled to peroxydase (GE Healthcare™ 934V) diluted 5,000× in PBS 1×, 1 % milk, 0.02 % Tween.

5.5. HeLa transfection and siRNA experiments

HeLa cells were cultivated in DMEM high glucose (4.5 g/l) and 10 % fetal bovine serum. Cultures were maintained at 37 °C and 5 % CO_2. Transfections were done in 6 well plates at 50 to 80 % of confluence. Each well was transfected with 4 μg of DNA using 10 μl of Lipofectamine transfection reagent (Invitrogen™), according to the supplier's instructions. RNA interference by siRNA was done in 6 well plates on cells seeded at 200 000 cells/well one day prior to tranfection. Each well was transfected with 50 pmol of RNAi using 7,5 μl of Lipofectamine RNAi MAX reagent (Invitrogen™), according to the supplier's instructions.

5.6. Live microscopy

Live imaging of the cells was done using a Zeiss LSM510 Confocor II Combi microscope with Argon laser and using LSM510 - FCS v.4.2 software.

PART III

6. Results

6.1. The phosphorylation of the NH_2-terminus of mouse CENP-A is essential for mitotic progression

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Summary

CENP-A is a histone variant that replaces histone H3 at the centromeres and marks them epigenetically. In human cells, the NH_2-terminus of CENP-A and its phosphorylation at serine 7 in mitosis has been reported to be crucial for the progression of mitosis. No phosphorylation of CENP-A in other metazoan species has been described. Here we show that the NH_2-terminus of CENP-A, but not its primary sequence and length, is required for mitosis in mouse embryonic cells (MEFs). Our data show that the mitotic defects resulting from the depletion of the endogenous CENP-A can be rescued when MEFs expressing a GFP-CENP-A mutant where the NH_2-terminus of CENP-A was swapped with the phosphorylatable tail of conventional histone H3. Conversely, no rescue was observed when the two phosphorylatable serines in the H3 tail mutant were replaced with alanines. Furthermore, a non-phosphorylatable fusion mutant of CENP-A where all seven serines in the amino-tail were replaced with alanines, was also unable to rescue the mitotic phenotype of CENP-A depleted cells. We also identify the first three serines (S15, S16 and S22) of the tail of CENP-A as potential sites for phosphorylation. Taken together, our results suggest that mitotic CENP-A phosphorylation is a common event in metazoan, which is essential for mitotic progression.

Introduction

Each cell, in addition to the core histones, expresses histone variants (van Holde, 1988). All histones, except H4, possess histone variants. Histone variants are non-allelic forms of conventional histones, they are expressed from distinct genes and exhibit strong

similarity to conventional histones (van Holde, 1988). Incorporation of histone variants into the nucleosome confers novel structural and functional properties to chromatin. Histone variants are key epigenetic factors involved in essentially all nuclear processes under both normal and pathological conditions (Boulard et al., 2007). For example, the histone variants macroH2A and H2A.Bbd, which belong to the H2A family, are implicated in transcriptional regulation (Angelov et al., 2003; Doyen et al., 2006a&b), while the histone variant H2B.FWT (from the H2B family) is likely involved in mitotic chromosome assembly (Boulard et al., 2006). Histone variants are also emerging players in cancer biology (Vardabasso et al., 2014).

The histone variant CENP-A replaces histone H3 at the centromeres and epigenetically marks the centromeres (Palmer et al., 1989; Palmer et al., 1990; Earnshaw et al., 1985). The CENP-A nucleosome exhibits distinct structure with highly flexible nucleosomal DNA ends (Tachiwana et al., 2011a; Roulland et al., 2016). This particular feature of the CENP-A nucleosome is required for assembly of active kinetochores as well as for mitotic fidelity (Roulland et al., 2016). The constitutive centromere-associated network (CCAN) consisting of 16 proteins (CENPs) is assembled onto CENP-A chromatin. CENP-C, a member of CCAN, is a protein with platform functions, required for operational kinetochore specification and spindle attachment (Perpelescu et al., 2011).

The amino-terminus of CENP-A is phosphorylated at serine 7 in mitosis in human cells (Zeitlin et al., 2001; Goutte-Gattat et al., 2013). The available data reveal that this mitotic phosphorylation of human CENP-A is required for kinetochore function. Mitotic CENP-A nucleosomal complexes contain CENP-C and phospho-binding 14-3-3 proteins. Direct interactions between the phosphorylated form of CENP-A and 14-3-3 proteins as well as between 14-3-3 proteins and CENP-C were demonstrated. The reported data revealed that 14-3-3 proteins are likely to act as “bridges,” linking phosphorylated CENP-A and CENP-C, which are necessary for the assembly and maintenance of active kinetochores (Goutte-Gattat et al., 2013).

The amino-terminal tail of CENP-A, in contrast to its structured (histone fold) domain, is highly variable between different species. The serine at position 7 present in human CENP-A, is not conserved amongst species. In addition, no phosphorylation of the

amino-terminal tail of CENP-A from other metazoan has been reported. This raises the question whether the reported data on the mitotic function of human CENP-A phosphorylation can be generalized or if it is restricted for human cells only.

Here we have analyzed the mitotic function of the CENP-A amino-terminus in MEF cells prepared from mouse embryos. Our data revealed that the phosphorylation of the tail of mouse CENP-A, but not its length nor its amino acid sequence, is required, for mitotic fidelity, as in the case for the human CENP-A. Since the amino-termini of CENP-A of distinct metazoan species contain one or more serines, we speculate that this could be a common evolutionary preserved mechanism that is required for chromosome segregation.

Results

The fusion of the amino-terminus of conventional histone H3 with the globular domain of CENP-A rescues the CENP-A depletion phenotype in MEF cells.

To analyze the role of the amino-tail of mouse CENP-A and its phosphorylation in mitosis we established conditional homozygous knockout/knockin (cKO/KI) mouse cell lines, where the endogenous CENP-A was replaced with a HA-FLAG-tagged CENP-A (Roulland et al., 2016). Using these mice we derived mouse embryonic fibroblasts (MEF), which, after immortalization, were infected with constructs expressing a tamoxifen inducible Cre-ERT2 recombinase and GFP-CENP-A (Figure 1a). Treatment with tamoxifen of these cells resulted in complete excision of the two CENP-A alleles and depletion of essentially all protein 6 days post-treatment (Figure 1c, d). The absence of CENP-A in control cells resulted, as expected and in agreement with the available data (Regnier et al., 2005; Roulland et al., 2016), in strong defects in both mitosis and interphase, including chromosome misalignments, chromosome bridges and micronuclei (Figure 1b, e, f). Quantification of the data showed that the ectopic expression of the GFP-CENP-A was sufficient to rescue this phenotype (Figure 1b, e, f).

In human HeLa cells, a swapped fusion of the tail of conventional H3 with the histone fold of human CENP-A was able to replace efficiently the endogenous CENP-A and rescue the mitotic phenotype resulting from its depletion (Goutte-Gattat et al., 2013). To test whether this is also the case in mouse cells, the immortalized MEFs were infected with both Cre-ERT2 recombinase and GFP-CENP-A mutant (GFP-H3-CENP-A, Figure 2a)

consisting of the tail of histone H3 fused to the histone fold of mouse CENP-A (Figure 2b, c).

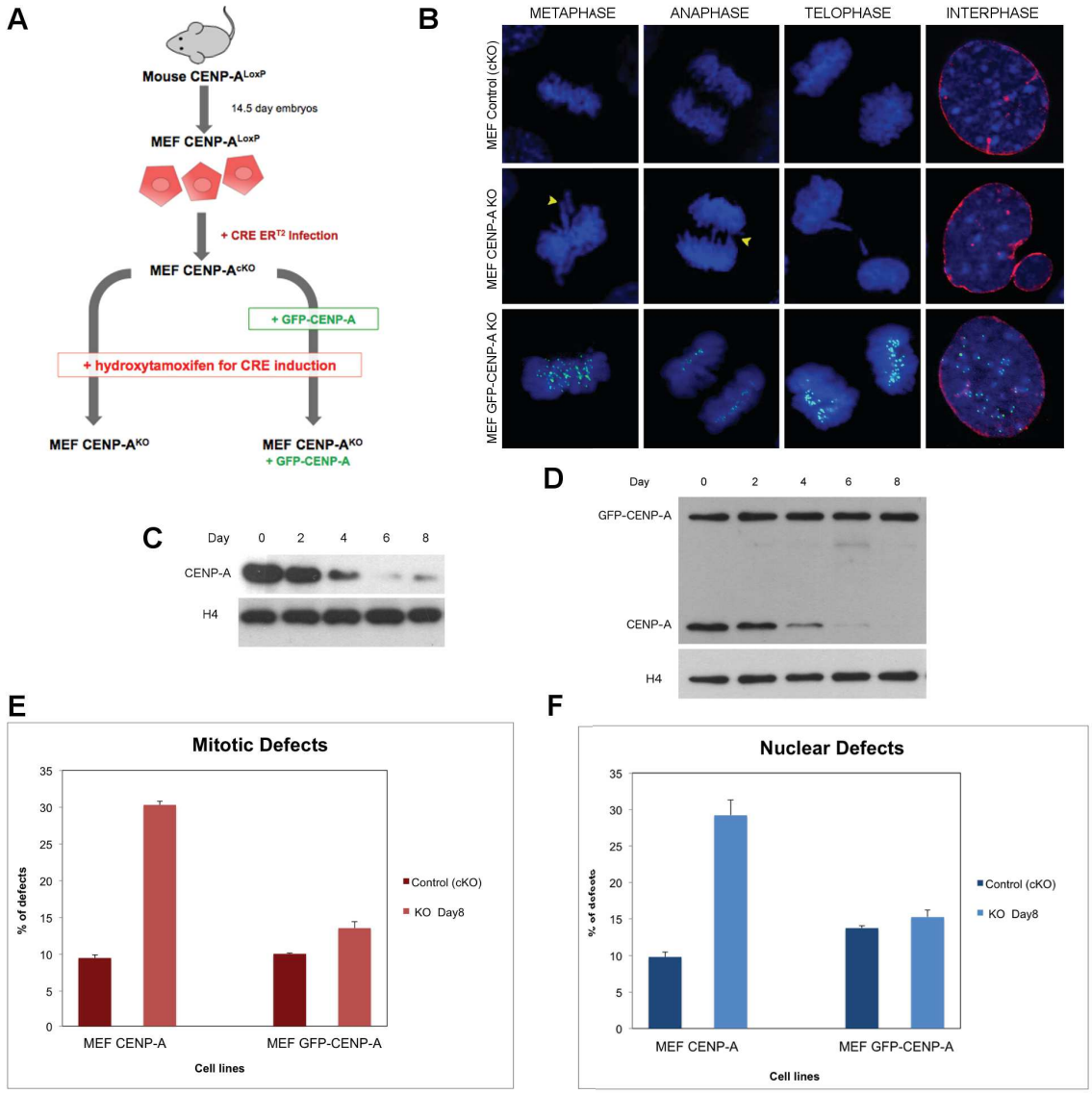

Figure 1. The replacement in MEFs of WT CENP-A by GFP-CENP-A at the centromere rescues the strong mitotic and nuclear defects caused by CENP-A depletion. (A) Schematics of the establishment of stable MEF cell lines and induction of CENP-A depletion. (B) Cell-cycle visualization of the effects of CENP-A depletion (8 days after the treatment with tamoxifen) in the indicated MEF lines. The first row shows the control CENP-A (Flox/Flox) MEFs before tamoxifen treatment, second row after. Western blots showing the HA-GFP-CENP-A fusion and endogenous HA-CENP-A in (C) control CENP-A (Flox/Flox) MEFs and (D) stable CENP-A (Flox/ Flox) MEFs expressing HA-GFP-CENP-A at different days after tamoxifen treatment. Quantification of the mitotic (E) and nuclear (F) defects in cell lines upon depletion of CENP-A, 8 days after tamoxifen treatment. The data are means and SEM from three different experiments.

This mouse fusion was able, as in the case of HeLa cells (Goutte-Gattat et al., 2013), to rescue the phenotype resulting from the depletion of CENP-A in MEF cells after six days of tamoxifen treatment (Figure 2c, f, g). Since the tail of H3 varies in primary sequence and in length compared to the tails of both human and mouse CENP-A (Figure

2b), this reveals that neither one of these two features of the CENP-A NH_2-terminus is determinant of CENP-A function.

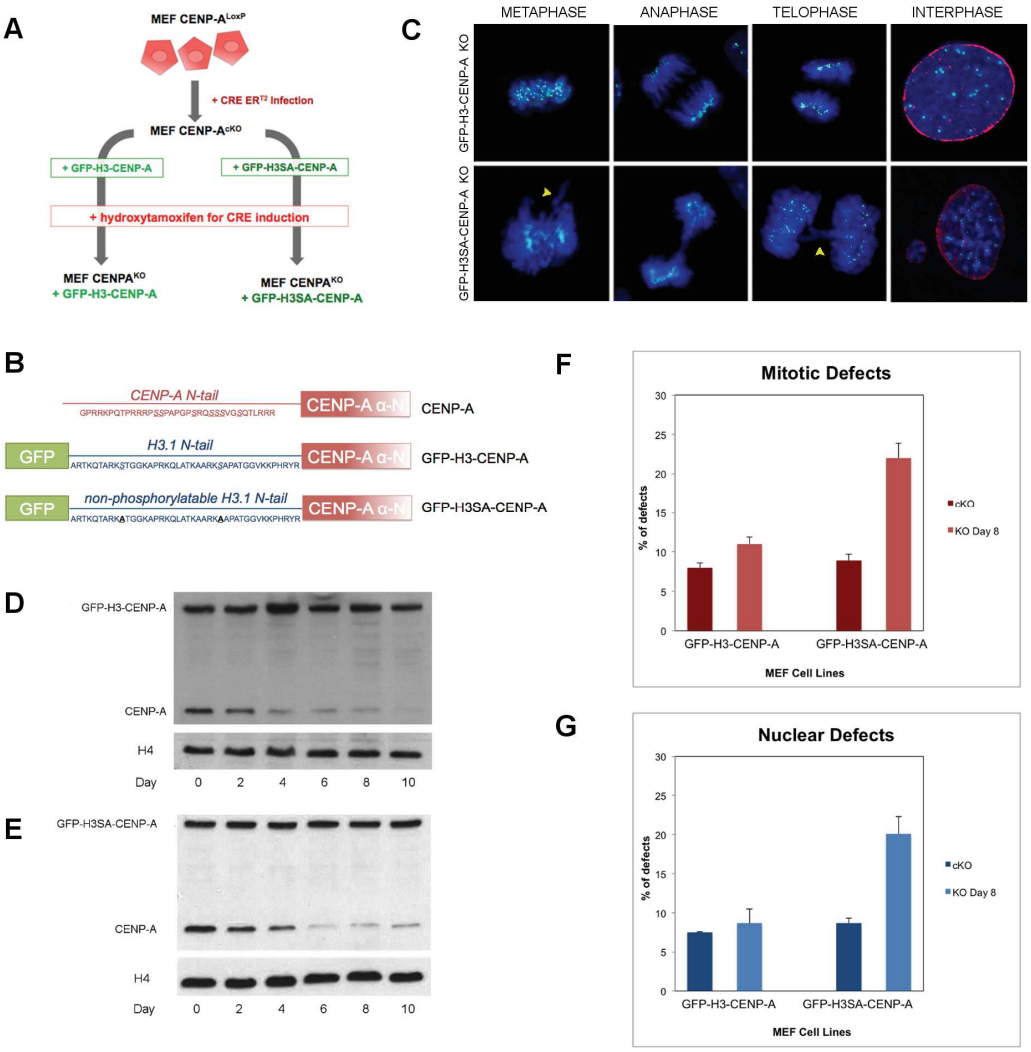

Figure 2. The replacement in MEFs of WT CENP-A by the swapped H3SA-CENP-A at the centromere results in strong mitotic and nuclear defects while H3-CENP-A rescues the phenotype. Schematics of (A) the experimental model (B) CENP-A swap constructs (C) Cell-cycle visualization of the effects of CENP-A depletion (8 days after the treatment with tamoxifen) in the indicated MEF lines. Western blots showing the HA-GFP-CENP-A fusions and endogenous HA-CENP-A in stable CENP-A (Flox/ Flox) MEFs expressing (D) HA-GFP-H3-CENP-A (E) HA-GFP-H3SA-CENP-A at different days after tamoxifen treatment. Quantification of the (F) mitotic and (G) nuclear defects in cell lines upon depletion of CENP-A, 8 days after tamoxifen treatment. The data are means and SEM from three different experiments.

The phosphorylation of the tail of CENP-A is essential for its mitotic function in MEFs

A non-phosphorylatable swapped fusion of the tail of conventional H3 (where the two mitotic phorphorytable serines 10 and 28, were substituted with alanines) with the globular domain of CENP-A was described to be unable to rescue CENP-A depletion phenotype in

HeLa cells (Goutte-Gattat et al., 2013). Our experiments reveal that this is also true for MEF cells. Indeed, CENP-A depleted MEFs upon 8 days of treatment with tamoxifen (Figure 2e), stably expressing non-phosphorytable mouse swapped CENP-A mutant (GFP-H3SA-CENP-A, Figure 2b), showed numerous mitotic and interphase defects (Figure 2c), comparable in amount to those of CENP-A depleted MEFs (Figure 2f, g compare with Figure 1e, f). Thus, the phosphorylation *per se*, and not the length of the CENP-A tail or its primary sequence, should be essential for mitotic progression.

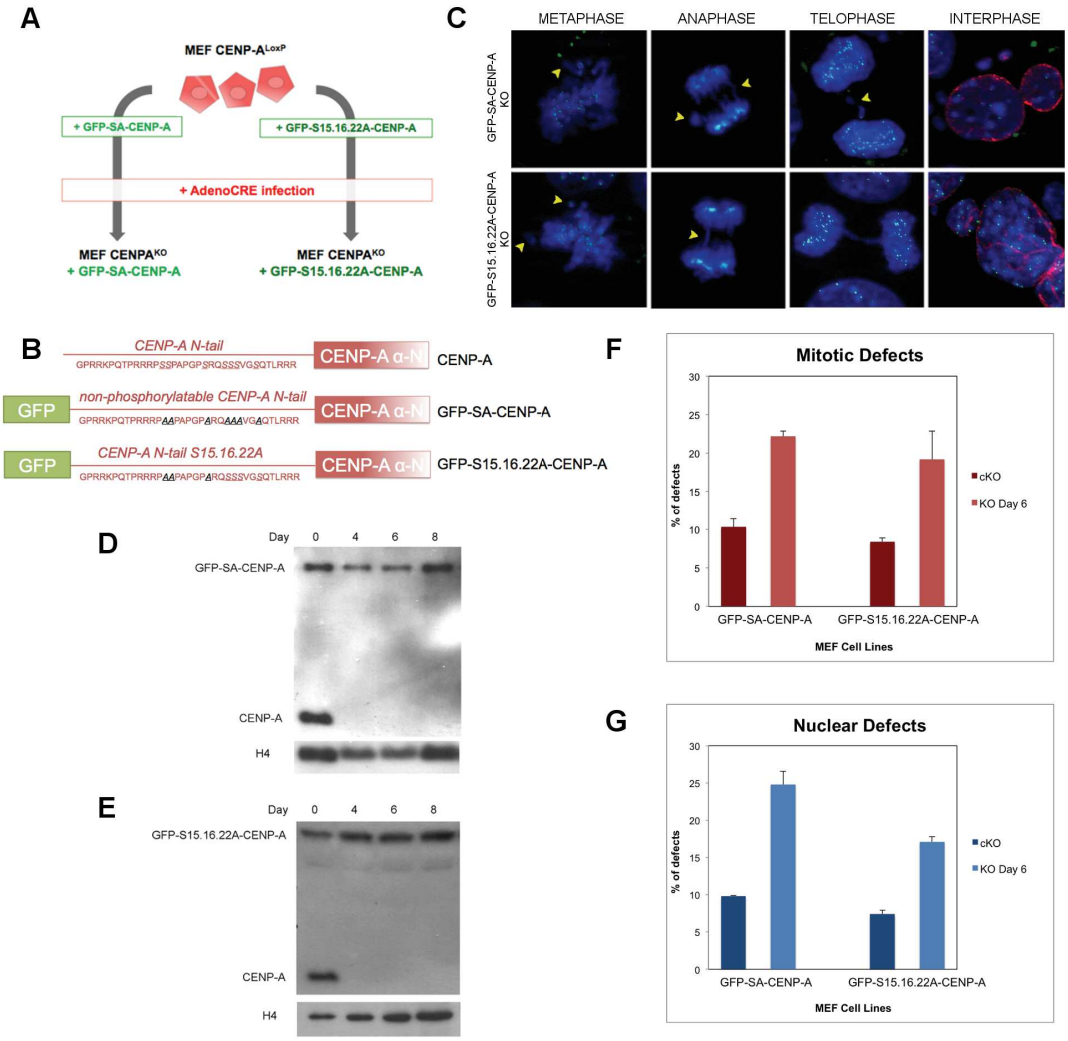

Figure 3. The replacement in MEFs of WT CENP-A by the non-phosphorylatable SA-CENP-A and S15.16.22A-CENP-A mutants at the centromere results in strong mitotic and nuclear defects. Schematics of (A) the experimental model (B) non-phosphorylatable CENP-A constructs (C) Cell-cycle visualization of the effects of CENP-A depletion (6 days after AdenoCRE infection) in the indicated MEF lines. Western blots showing the HA-GFP-CENP-A fusions and endogenous HA-CENP-A in stable CENP-A (Flox/ Flox) MEFs expressing (D) HA-GFP-SA-CENP-A (E) HA-GFP-S15.16.22A-CENP-A at different days after AdenoCRE infection. Quantification of the (F) mitotic and (G) nuclear defects in cell lines upon depletion of CENP-A, 6 days after AdenoCRE infection. The data are means and SEM from three different experiments.

If this is the case, then a non-phosphorylatable CENP-A tail mutant should be unable to function as the native protein and will be unable to rescue the CENP-A depleted MEF's phenotype. The tail of mouse CENP-A contains seven serines along its length (Figure 3b). We constructed a GFP-CENP-A mutant (GFP-SA-CENP-A), where all 7 serines were substituted to alanines (Figure 3b) and established stable MEF lines expressing it. After depletion of endogenous CENP-A upon AdenoCre recombinase infection (Figure 3d, e), the cell phenotype was analyzed. As seen (Figure 3c, f, g), the non-phosphorylatable GFP-SA-CENP-A mutant was unable to rescue the MEF phenotype and these cells behaved mitotically as the CENP-A depleted MEFs (Figure 3c, f, g). We thus conclude that mitotic phosphorylation of mouse CENP-A is required for mitotis.

We next sought to determine the potentially phosphorylatable serine(s) of the CENP-A tail, which is responsible for the mitotic function. To address this we used a mutant (GFP-S15.16.22A-CENP-A) where the first three serines (S15, S16 and S22, Figure 3b) were substituted with alanines and established stable MEFs (Figure a). This mutant behaved exactly as the GFP-SA-CENP-A mutant with all seven serines mutated to alanines, i.e. no rescue was observed in the stable CENP-A depleted cells expressing this mutant (Figure 3 c, d, f, g). These data reveal that the phosphorylation of S15, S16 or S22, in combination or alone, is necessary for proper mitotic progression.

The phosphorylation of mouse CENP-A is required for CENP-C localization at centromeres.

In human cells, the localization of the kinetochore platform protein CENP-C is dependent on CENP-A phosphorylation. Bearing in mind the high similarity of the role of CENP-A phosphorylation in mouse and in human cells, one should expect the same behavior of mouse CENP-C. We addressed this question in a series of experiments where we used CENP-A depleted MEFs stably expressing RFP-CENP-C fusion with either one of the studied mutants (Figure 4a). RFP-CENP-C continued to colocalize with GFP-H3-CENP-A at centromeres in cells depleted of CENP-A. In contrast, the amount of RFP-CENP-C was significantly reduced at centromeres in cells expressing either one of the three non-phohphorylatable mutants (GFP-H3SA-CENP-A, GFP-SA-CENP-A, or GFP-SA-CENP-A) upon CENP-A depletion (Figure 4b, c). These results show, that the phosphorylation of CENP-A is required, as in HeLa cells, for the cetromeric localization of CENP-C.

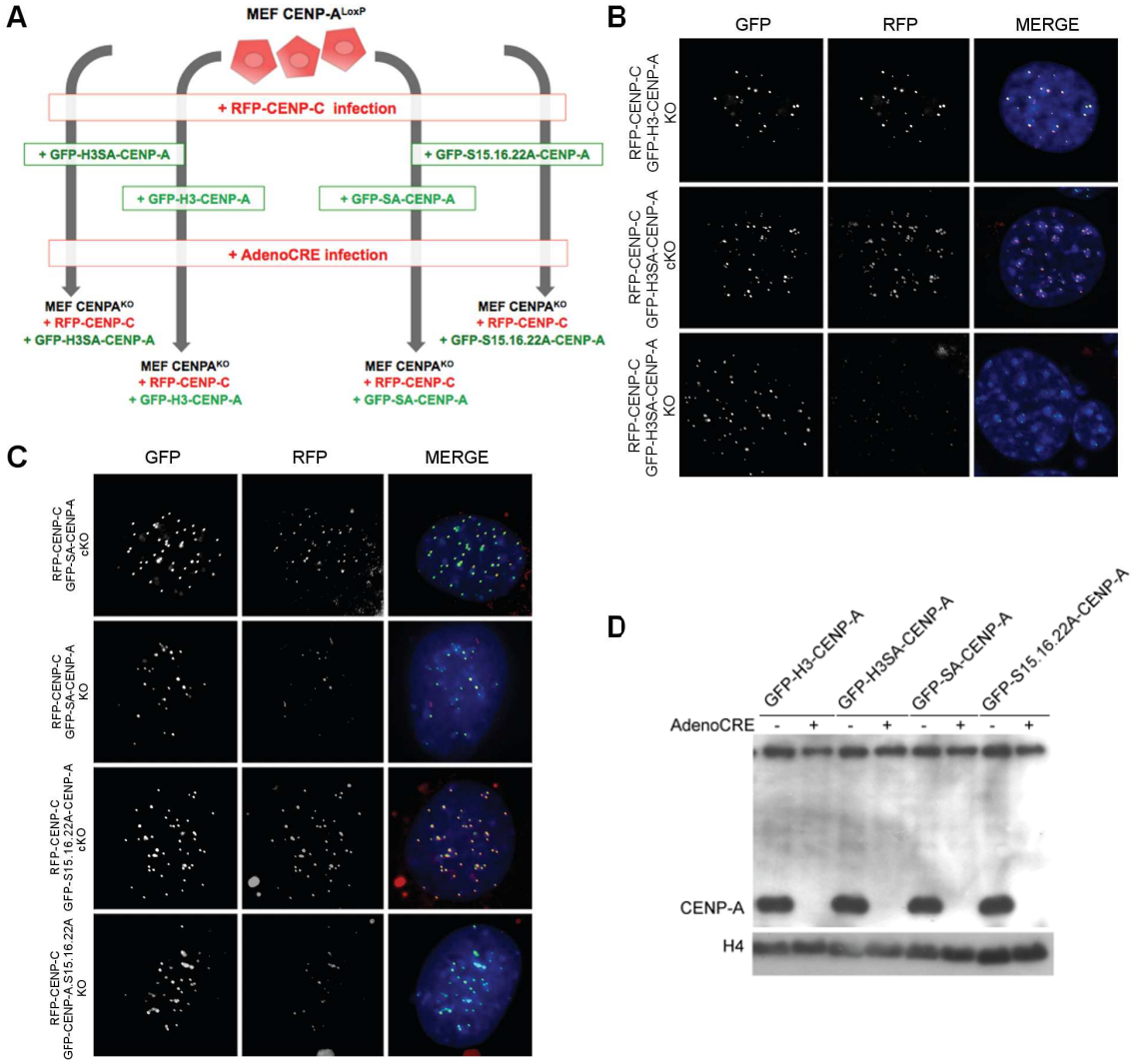

Figure 4. The incorporation of non-phosphorylatable mutants into the centromeres affects the localization of CENP-C in CENP-A depleted MEFs (A) Schematics of the experimental model (B) Localization of RFP-CENP-C in MEFs stably expressing GFP-H3-CENP-A after CENP-A depletion (first row) and GFP-H3SA-CENP-A before (second row) and after CENP-A depletion (last row) (C) Localization of RFP-CENP-C in MEFs stably expressing GFP-SA-CENP-A before (first row) and after CENP-A depletion (second row) and GFP-S15.16.22A-CENP-A before (third row) and after CENP-A depletion (last row) (D) Western blots showing the HA-GFP-CENP-A fusions and endogenous HA-CENP-A in stable CENP-A (Flox/ Flox) MEFs expressing RFP-CENP-C before and after AdenoCRE infection.

Discussion

The mitotic phosphorylation of CENP-A tail at serine 7 was reported only in human cells (Zeitlin et al., 2001; Goutte-Gattat et al., 2013). The available data showed, however, that this phosphorylation is an important event and it is required for proper chromosome segregation and mitotic progression (Goutte-Gattat et al., 2013). This suggests that mitotic phosphorylation of the CENP-A amino-tail should be observed in other metazoans and that it could have a role similar to that in human cells. Here we have tested this hypothesis.

Since no specific antibodies against mouse CENP-A and its potentially phosphorylated mitotic form(s) existed, we have used an indirect approach. We first showed that the non-phosphorylatable swapped mutant GFP-H3SA-CENP-A, in contrast to its phosphorytable form GFP-H3-CENP-A, has lost, as in HeLa cells, its capacity to rescue the mitotic phenotype of MEFs depleted of endogenous CENP-A. Since the tail of H3 is distinct in both length and sequence from that of CENP-A, these results strongly suggest that: (i) neither the length nor the primary amino-acid sequence of the amino-terminus of CENP-A is crucial for its function in mitosis, and (ii) the mere phosphorylation of CENP-A amino-tail is essential for its mitotic function. In agreement with this claim, we found that the non-phosphorylatable GFP-SA-CENP-A mutant behaved as the non-phosphorylatable swapped mutant GFP-H3SA-CENP-A and was unable to rescue the mitotic phenotype of cells depleted of CENP-A. Our data further identified among the seven serines present in the CENP-A tail, the first three of them, namely S15, S16 and S22, as potential sites for phosphorylation, since the mutant GFP-S15.16.22A (where these three serines were substututed with alanines) behaved as the completely non-phosphorylatable GFP-SA-CENP-A and was unable to rescue the mitotic defects of MEFs depleted of CENP-A. In addition, the expression of either GFP-S15.16.22A or GFP-SA-CENP-A in CENP-A depleted MEF's did not lead, in contrast to the phosphorylatable counterparts, to the rescue of the centromeric localization of CENP-C.

In summary, our data reveals that the mouse CENP-A, as human CENP-A, appears to be phosphorylated during mitosis. This phosphorylation plays an essential function in chromosome segregation. We speculate that mouse mitotically phosphorylated CENP-A is, as in HeLa cells, a target for the phosphobinding proteins 14-3-3 that could act as specific mitotic "bridges," linking phosphorylated CENP-A and CENP-C necessary for mitotic progression (Goutte-Gattat et al., 2013). The 14-3-3 proteins would recognize and bind to an accessible phosphate group in the CENP-A tail and thus, no specific position of the phosphorylatable serine should be required. This could explain the raison why the tails of the CENP-A from different species contain serines, located at different positions. We speculate that the first serine(s) within the tails of the CENP-A proteins in metazoan would be, due to their higher accessibility, phosphorylated at the onset of mitosis.

Materials and Methods

CENP-A KI-cKO Mouse Line The CENP-A cKO/KI mouse line was established by using standard mouse genetic approaches. In this line, the CENP-A gene was replaced by a double tagged FLAG-HA-CENP-A fusion. The targeting vector of the generated cKO/ KI FLAG-HA tagged CENP-Aflox/flox mice flanked exon 1 of the murine CENP-A gene with LoxP recombination sites.

MEF Cell Lines MEFs were isolated and cultured from day 14,5 embryos of the CENP-A cKO/KI mice and were immortalized via SV40 infection. Cells were cultivated in DMEM high glucose (4.5 g/l) and 10 % fetal bovine serum and 1% penicillin and streptomycin at 37 °C and 5 % CO2.

CRE recombinase expression for CENP-A knock-out was assured by MSCV CreERT2 (Addgene #22776) retroviral infection fallowed by 2μM (Z)-4-Hydroxytamoxifen (Sigma-Aldrich) treatment or by adenovirus infection (Ad-CMV-iCre, Vector Biolabs, Philadelphia, PA).

The construct H3-CENP-A was obtained by replacing the first 35 amino acids of the CENP-A amino tail with the first 42 amino acids of that of histone H3.1. H3SA-CENP-A is the same as H3-CENP-A construct except for S10 and S28 mutated to alanine. The construct SA-CENP-A has all seven serine residues in the mouse CENP-A amino tail mutated into alanines while S15.16.22A-CENP-A has only the first three serine residues. All CENP-A constructs were fused at the amino terminus with HA-GFP and then cloned into retroviral pCLMFG vector. All cell lines resulting from these infections were FACSed (BD FACS Aria Cell Sorting System) to obtain cell lines with homogenous expression of the exogenous CENP-A at near endogenous expression level.

CENP-C sequence was fused at the amino terminus with RFP and then cloned into pQCXIH vector (Clontech). MEF cKO cells were initially infected with CENP-C then these cells were infected with the GFP-CENP-A constructs.

Immunocytochemistry Cells were fixed with 4% formalin solution (Sigma-Aldrich) for 20 minutes at 37 °C then washed 3 times with PBS. Cells were then permeabilized with 0.02 % Triton for 3 minutes. The coverslips were then successively incubated for an hour at 37 °C with primary anti-lamin (1:1000, Santa Cruz biotechnology) and secondary anti-goat

Cy3 (1:300, SantaCruz) antibodies. DNA was stained in the presence of 1.5 µg of Hoechst 33342.

The slides were later observed and imaged using a Zeiss Axiovision epifluorescence microscope with Plan-Apochromat 63× objective. GFP, cyanine-3, and Hoechst 33342 were used as fluorochromes. Images were acquired with a Zeiss Axiocam camera piloted with the Zeiss Axiovision software.

The rate of defects was calculated from a sample of 200 ± 25 mitotic cells for mitotic defects and 400 ± 25 nuclei for nuclear defects.

Nuclear Isolation for Immunoblotting Cells were pelleted then resuspended in 250 µl of Buffer B (NaCl 15 mM, Tris HCl pH 7.5 15 mM, KCl 60 mM, sucrose 11 %, EDTA 2 mM, EGTA 0.5 mM, DTT 1 mM, spermine 0.2 mM, spermidine 0.65 mM, PMSF 0.5 mM). Next 250 µl of Lysis Buffer (Buffer B with 4 % NP-40) was added and the suspension was incubated 5 minutes on ice. The cell lysate was then successively centrifugated twice at 13,200 rpm for 5 minutes. After each centrifugation, the pellet was resuspended respectively in: 250 µl of Buffer D (Buffer B without EDTA and EGTA) and finally 150 µl of PBS 1× with 1× Laemmli buffer. Samples where then sonicated at 200 J/W.s and used for immunoblotting.

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References

Angelov, D.; Molla, A.; Perche, P. Y.; Hans, F.; Côté, J.; Khochbin, S.; Bouvet, P.; Dimitrov, S., The histone variant macroH2A interferes with transcription factor binding and SWI/SNF nucleosome remodeling. *Mol Cell* **2003,** *11* (4), 1033-41.

Ausio, J.; Van Holde, K. E., The histones of the sperm of Spisula solidissima include a novel, cysteine-containing H-1 histone. *Cell Differ* **1988,** *23* (3), 175-89.

Boulard, M.; Bouvet, P.; Kundu, T. K.; Dimitrov, S., Histone variant nucleosomes: structure, function and implication in disease. *Subcell Biochem* **2007,** *41*, 71-89.

Boulard, M.; Gautier, T.; Mbele, G. O.; Gerson, V.; Hamiche, A.; Angelov, D.; Bouvet, P.; Dimitrov, S., The NH2 tail of the novel histone variant H2BFWT exhibits properties distinct from conventional H2B with respect to the assembly of mitotic chromosomes. *Mol Cell Biol* **2006,** *26* (4), 1518-26.

Doyen, C. M.; An, W.; Angelov, D.; Bondarenko, V.; Mietton, F.; Studitsky, V. M.; Hamiche, A.; Roeder, R. G.; Bouvet, P.; Dimitrov, S., Mechanism of polymerase II transcription repression by the histone variant macroH2A. *Mol Cell Biol* **2006,** *26* (3), 1156-64.

Doyen, C. M.; Montel, F.; Gautier, T.; Menoni, H.; Claudet, C.; Delacour-Larose, M.; Angelov, D.; Hamiche, A.; Bednar, J.; Faivre-Moskalenko, C.; Bouvet, P.; Dimitrov, S., Dissection of the unusual structural and functional properties of the variant H2A.Bbd nucleosome. *EMBO J* **2006,** *25* (18), 4234-44.

Earnshaw, W. C.; Migeon, B. R., Three related centromere proteins are absent from the inactive centromere of a stable isodicentric chromosome. *Chromosoma* **1985,** *92* (4), 290-6.

Goutte-Gattat, D.; Shuaib, M.; Ouararhni, K.; Gautier, T.; Skoufias, D. A.; Hamiche, A.; Dimitrov, S., Phosphorylation of the CENP-A amino-terminus in mitotic centromeric chromatin is required for kinetochore function. *Proc Natl Acad Sci U S A* **2013,** *110* (21), 8579-84.

Palmer, D. K.; O'Day, K.; Margolis, R. L., Biochemical analysis of CENP-A, a centromeric protein with histone-like properties. *Prog Clin Biol Res* **1989,** *318*, 61-72.
Palmer, D. K.; O'Day, K.; Margolis, R. L., The centromere specific histone CENP-A is selectively retained in discrete foci in mammalian sperm nuclei. *Chromosoma* **1990,** *100* (1), 32-6.

Perpelescu, M.; Fukagawa, T., The ABCs of CENPs. *Chromosoma* **2011,** *120* (5), 425-46.

Régnier, V.; Vagnarelli, P.; Fukagawa, T.; Zerjal, T.; Burns, E.; Trouche, D.; Earnshaw, W.; Brown, W., CENP-A is required for accurate chromosome segregation and sustained kinetochore association of BubR1. *Mol Cell Biol* **2005,** *25* (10), 3967-81.

Roulland, Y.; Ouararhni, K.; Naidenov, M.; Ramos, L.; Shuaib, M.; Syed, S. H.; Lone, I. N.; Boopathi, R.; Fontaine, E.; Papai, G.; Tachiwana, H.; Gautier, T.; Skoufias, D.;

Padmanabhan, K.; Bednar, J.; Kurumizaka, H.; Schultz, P.; Angelov, D.; Hamiche, A.; Dimitrov, S., The Flexible Ends of CENP-A Nucleosome Are Required for Mitotic Fidelity. *Mol Cell* **2016,** *63* (4), 674-85.

Tachiwana, H.; Kagawa, W.; Shiga, T.; Osakabe, A.; Miya, Y.; Saito, K.; Hayashi-Takanaka, Y.; Oda, T.; Sato, M.; Park, S. Y.; Kimura, H.; Kurumizaka, H., Crystal structure of the human centromeric nucleosome containing CENP-A. *Nature* **2011,** *476* (7359), 232-5.

Vardabasso, C.; Hasson, D.; Ratnakumar, K.; Chung, C. Y.; Duarte, L. F.; Bernstein, E., Histone variants: emerging players in cancer biology. *Cell Mol Life Sci* **2014,** *71* (3), 379-404.

Zeitlin, S. G.; Shelby, R. D.; Sullivan, K. F., CENP-A is phosphorylated by Aurora B kinase and plays an unexpected role in completion of cytokinesis. *J Cell Biol* **2001,** *155* (7), 1147-57.

6.2. Investigating the function of the amino-terminal domain of CENP-A during mitosis using the "Hara-kiri" approach

We have shown that the CENP-A amino-terminus plays a major function in mitosis by its ability to be phosphorylated, but the data is indirect. To unambiguously tie this function to mitosis, it is necessary to remove the CENP-A tail only during mitosis but no techniques are currently available to achieve this.

We have devised a new method that we call the "Hara-kiri" approach in order to answer the above question in human HeLa cells, where the amino terminal domain has been shown to be important for proper chromosome segregation (Goutte-Gattat et al, 2013). In this approach, we fuse to CENP-A a specific protease we named "Katana" and we insert within the amino-tail of CENP-A the target cleavage site of the Katana protease (Figure 6.1). The cleavage site is specifically recognized by the Katana protease and does not naturally exist in the human proteome. Once synthesized, we expect this fusion protein to be cleaved by its own proteolytic activity, thus resulting with CENP-A protein lacking the amino-terminus. The activity of the Katana protease can be delayed by cultivating cells in the presence of a specific, reversible inhibitor, thereby allowing us to control the time that the NH_2-tail should be cleaved (Figure 6.1).

We can thus apply the hara-kiri approach to synchronized cells grown in presence of the inhibitor. Then, by removing the inhibitor prior to mitotic entry, we should be able to eliminate the NH_2-tail and detect whether mitotic defects occur as a direct result of the absence of the amino-terminus of CENP-A.

One concern while studying the effects of tailless CENP-A is that it's a recessive mutant, as long as wild type CENP-A is expressed in cells, no phenotype deriving from the expression of the tailless mutant will appear. To overcome this, endogenous CENP-A expression will be knocked down by siRNA. The CENP-A sequence that we have chosen for the Hara-kiri approach is a silent resistant sequence so it will not be affected by the siRNA mediated silencing.

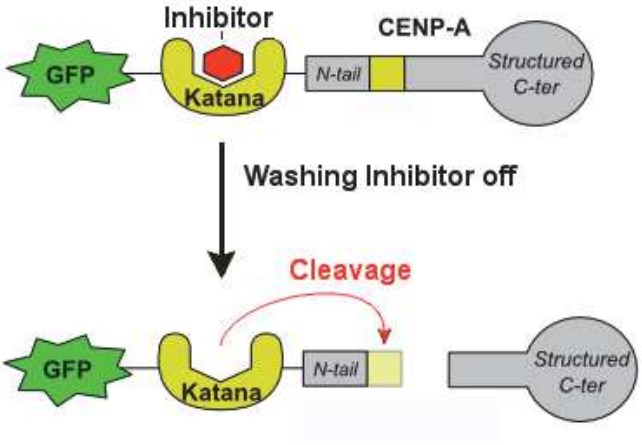


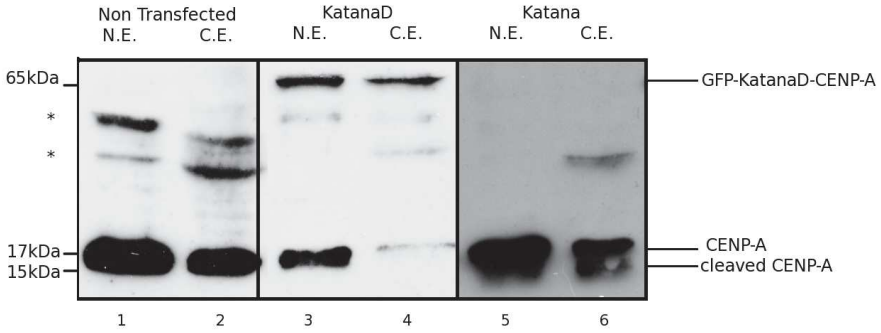
Figure 6.1 The "Hara-kiri" approach: Removal of the inhibitor from the culture medium activates the protease, the CENP-A amino-terminus is cleaved.

Expression and cleavage of the Katana fusion proteins

Initially we needed to verify whether the Katana fusion proteins could be expressed in cells and determine if the self-cleavage of the protein was possible. For this HeLa cells were transfected using both dead and active Katana vectors and grown for 54 hours post-transfection. Then we performed nuclear extractions on the transfected cells followed by Western blot analysis using an anti-CENP-A antibody directed against the C-terminal domain of CENP-A.

Non-transfected cells were used as a control. We detected endogenous CENP-A in both nuclear (Figure 6.2, lane 1) and cytoplasmic extracts (lane 2). The nuclear extract was more enriched as expected since CENP-A is a nuclear protein. In cells transfected with the dead Katana vector, a 65 kDa band was detectable in both the nuclear and the cytoplasmic extracts. This band was at the expected size for the whole GFP-KatanaD-nCENP-A fusion protein (lanes 3 and 4), confirming that the fusion could in fact be expressed in cells.

In cells transfected with the active Katana vector we detected a 15 kDa band, just below the endogenous CENP-A; this corresponds to the expected size of the C-terminal fragment of the cleaved CENP-A and allows us to conclude that self-cleavage did occur in these cells. The total absence of the intact GFP-Katana-nCENP-A fusion (note the absence of a 65 kDa band in lanes 5 and 6) indicates that all synthesized proteins were cleaved and reveals the efficiency of the cleavage. We can also remark that the cleaved fragment is detected in both nuclear and cytoplasmic extracts, indicating that the cleavage occurred rapidly after protein synthesis, before the translocation to the nucleus.



6.2 The Katana fusions are expressed and the active form is cleaved in cells. HeLa cells were transfected with the indicated Katana vectors, nuclear extractions were performed 54 hours post transfection. Western-blot analysis was performed with an anti-CENP-A antibody recognizing the C-terminal domain of CENP-A on both nuclear (N.E.) and cytoplasmic extracts (C.E.). Endogenous CENP-A is detected in all lanes at 17kDa. In cells transfected with the GFP- KatanaD-nCENP-A vector a 65kDa band corresponding to the size of the fusion is detected (lanes 3–4). In cells transfected with the GFP-Katana-nCENP-A vector a 15kDa band corresponding to the size of the C-terminal fragment of the cleaved fusion is detected (lanes 5–6). Asterisks show non-specific bands.

Localization of the inactive fusion protein

We next wanted to know whether CENP-A could still be recruited to the centromeres while fused to the Katana protease, the GFP as well as containing the Katana target site within its NH_2-terminal domain. For this we transfected cells with the vector construct expressing the dead form of Katana and performed immunofluorescence staining with a CREST antibody that marks the centromeres; we used the natural fluorescence of the GFP to detect the inactive fusion protein.

In interphase, we could see that the GFP signal colocalized with the CREST staining at the centromeres (Figure 6.3, upper row). In mitosis (middle and lower rows), the CREST staining was larger than the GFP signal, with the GFP signal overlapping only on its edges; this was expected, due to the fact that the CREST antibody recognizes proteins located in the whole centromere while CENP-A location is restricted to the outer centromere. Overall, we concluded that the GFP-KatanaD-nCENP-A fusion protein can be recruited to centromeric chromatin and can localize as the endogenous CENP-A.

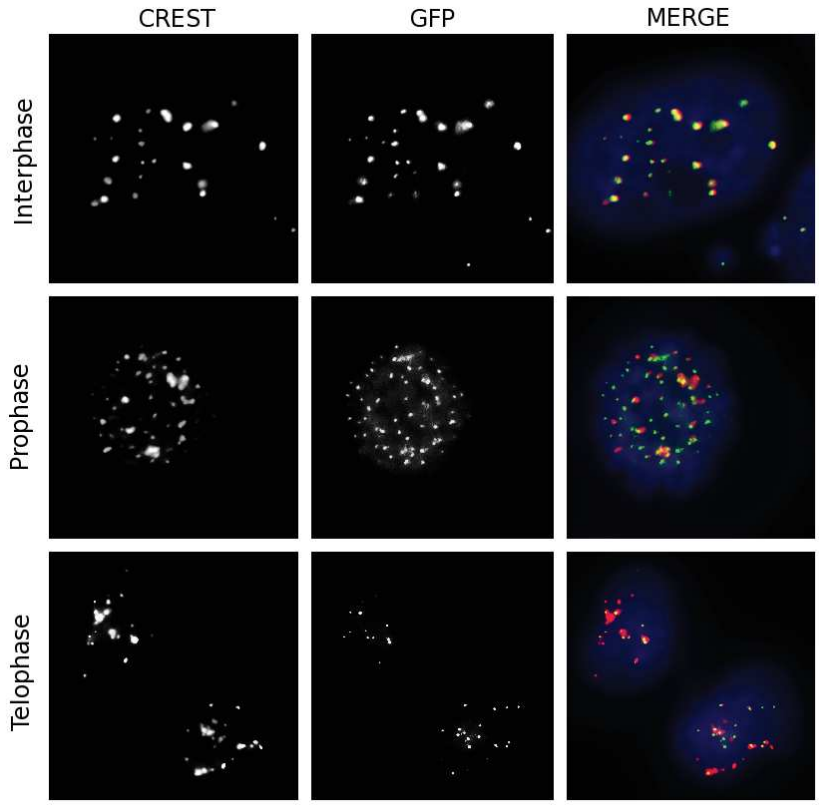


Figure 6.3 The inactive Katana fusion protein localizes to the centromeres. HeLa cells transfected with the GFP-KatanaD-nCENP-A vector were submitted to immunofluorescence analysis with a CREST antibody that marks the centromeres. Hoechst was used to stain DNA and GFP to detect the fusion protein. The GFP signal was found to colocalize with the centromeric CREST signal in interphase (upper row), in prophase (middle row) and in telophase (lower row).

Inhibition and Cleavage of the active Katana fusion protein

We finally wanted to check our ability to prevent the cleavage of the active Katana fusion protein. For this cells were transfected with the active Katana vector and grown in the presence or the absence of the Katana inhibitor. By immunofluorescence we could see that in cells that were grown in absence of the inhibitor, no GFP signal was detected at the centromeres (Figure 6.4, middle row). As expected, without the inhibitor the fusion protein undergoes cleavage before being translocated to the nucleus, so the cleaved CENP-A is no longer fused to the GFP and is thus undetectable.

In the presence of 9 μM of Katana inhibitor, we expect the GFP-Katana-nCENP-A fusion protein to stay intact and behave like the dead Katana fusion. This is what we observed (Figure 6.4, lower row, compare to upper row); the GFP signal colocalized with the CREST staining, revealing that the GFP remained attached to the globular domain of

CENP-A. This allows us to conclude that growing the cells in the presence of 9 μM of inhibitor was sufficient to inhibit the protease and prevent the self-cleavage of the fusion protein in cells.

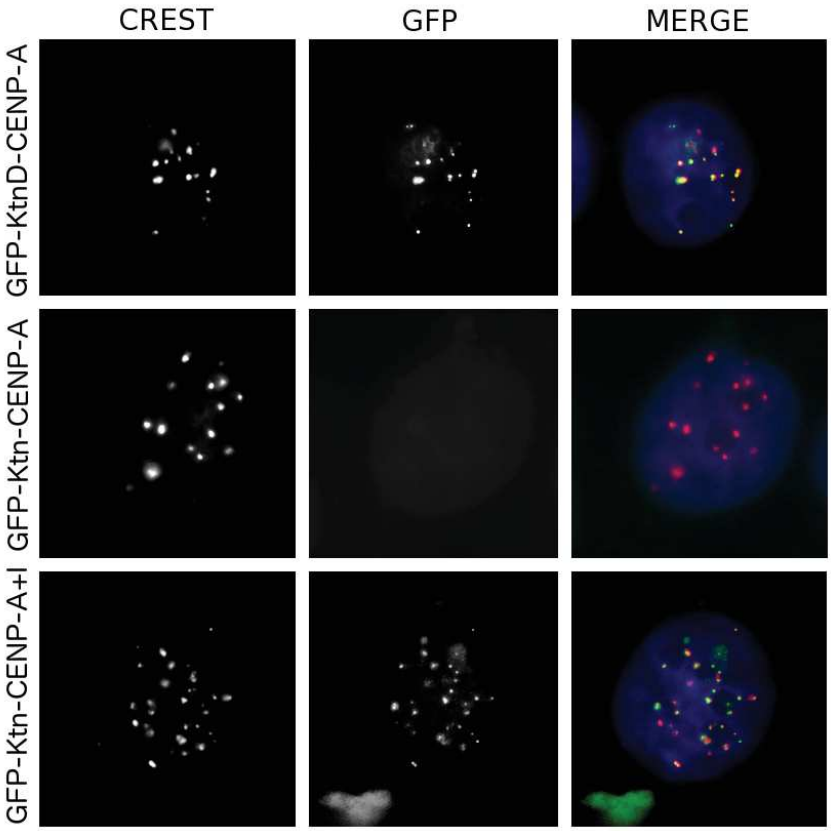


Figure 6.4. The inhibitor prevents the cleavage of the Katana fusion protein. HeLa cells transfected with the GFP-KatanaD-nCENP-A vector (upper row) or the GFP-Katana-nCENP-A vector were submitted to immunofluorescence analysis as before. Cells transfected with the active Katana vector and grown without the Katana inhibitor (middle row) exhibited no centromeric GFP signal. Cells grown with 9 μM of inhibitor (lower row) showed the same phenotype as cells transfected with the inactive Katana fusion (upper row).

The final step before applying the hara-kiri approach to study the amino terminal function of CENP-A was to confirm whether the GFP-Katana-nCENP-A fusion could be cleaved once it was already incorporated into centromeric chromatin. We were unable to achieve cleavage since the GFP signal remained at the centromeres after inhibitor removal (data not shown). We postulated that within the crowded centromeric environment the protease could not access its target and we were able to overcome this problem by introducing two additional cleavage sites to the amino tail. Cells were transfected with the GFP-Katana-nnnCENP-A vector and grown in the presence of the inhibitor. Coverslips were then fixed at: 0, 15, 30 and 60 minutes after inhibitor removal. Washing out of the inhibitor resulted in GFP-fluorescence progressively disappearing from the centromeres,

suggesting that CENP-A fusion is cleaved at its NH_2-terminus as the inhibition of the Katana protease is reversed. One hour after inhibitor removal the GFP signal was diffused (Figure 6.5).

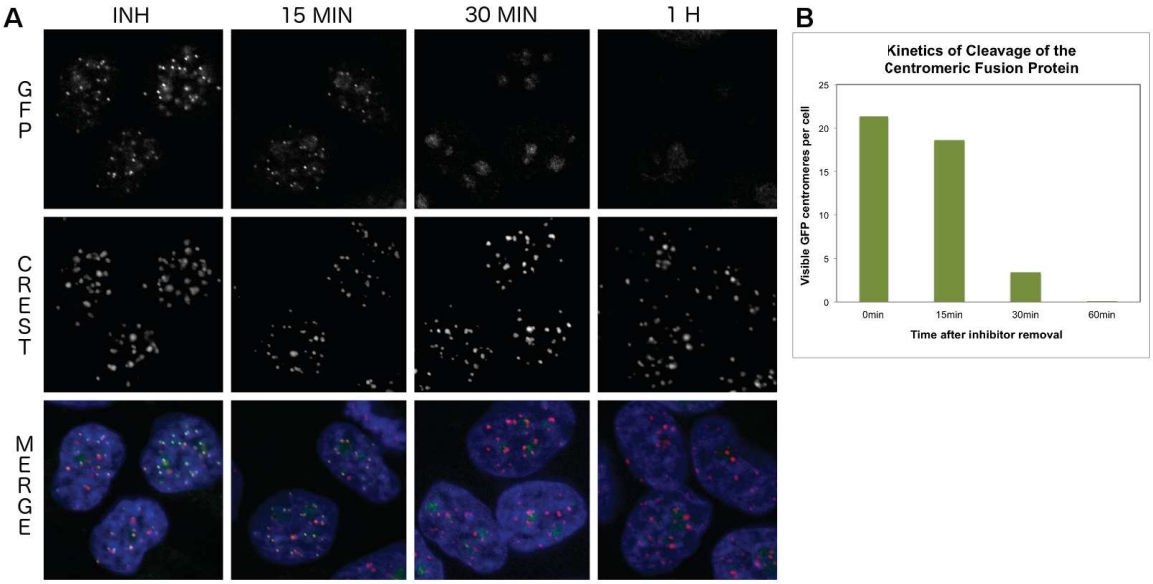


Figure 6.5. The centromeric Katana fusion protein is cleaved upon inhibitor removal. HeLa cells transfected with the GFP-Katana-nnnCENP-A vector were submitted to immunofluorescence analysis with CREST antibody after 0, 15, 30 and 60 minutes after inhibitor removal. A) The GFP signal found to colocalize with the centromeric CREST signal decreased progressively after inhibitor removal (15 and 30 minutes) and was defused at 60 minutes. B) Total amount of GFP signal decreased after inhibitor removal and was near undetectable after 60 minutes.

Validation of the Hara-kiri approach in stable cell lines expressing the fusion protein

HeLa cell lines stably expressing the GFP-Katana-nnnCENP-A were generated. Monoclonal cell lines expressing near endogenous levels of the CENP-A fusion protein were selected, cautious to the fact that CENP-A overexpression results in partial misincorporation of CENP-A outside of the centromeres (Van Hooser et al., 2001) and can in itself cause mitotic defects. Western blot analysis showed that the exogenous CENP-A fusion protein was expressed at near endogenous levels and was cleaved in absence of the inhibitor (Figure 6.6a).

Additionally the cleavage of the CENP-A fusion protein was observed by live video-microscopy, the cleavage took place in similar fashion to that was observed by immunofluorescence analysis as the GFP signal gradually decreased and was diffused within sixty minutes (Figure 6.6b). This allowed us to validate the Hara-kiri model and

proceed to applying the approach to the investigation of the function of the amino-terminal domain of CENP-A.

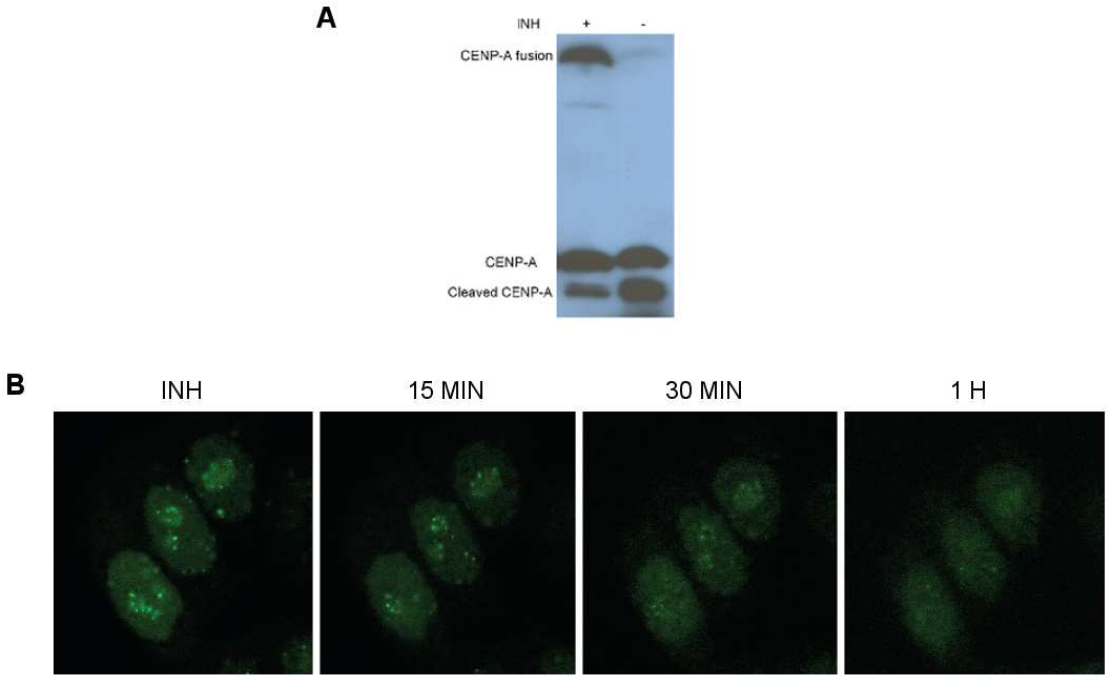


Figure 6.6 The centromeric Katana fusion protein is cleaved upon inhibitor removal in stable cell lines. A) Western blot of HeLa cell line stably expressing the GFP-Katana-nnnCENP-A fusion protein: in presence of the inhibitor the fusion protein is intact (lane 1) and is cleaved in the absence of the inhibitor (lane 2). B) Time-lapse of HeLa cell line stably expressing the GFP-Katana-nnnCENP-A at 0, 15, 30 and 60 minutes after inhibitor removal. The GFP signal is decreased progressively after inhibitor removal (15 and 30 minutes) and near undetectable after 60 minutes.

Removal of the CENP-A tail before mitotic entry results in increased mitotic defects

Stable cell lines expressing the GFP-Katana-nnnCENP-A fusion were grown for three days in the presence of the inhibitor. Cells were then synchronized by double thymidine block and endogenous CENP-A was knocked-down using siRNA (Figure 6.7b). The inhibitor was removed from the cell culture medium 60 minutes before cells entered mitosis and cells were fixed 30 minutes after mitotic entry. Thus, the pool of centromeric CENP-A in cells has lost their amino-terminal domains just as the cells enter mitosis.

The cleavage of the GFP-Katana-CENP-A fusion at the onset of mitosis led to increased mitotic defects in cells depleted of endogenous CENP-A such as the presence of misaligned chromosomes in metaphase, lagging chromosomes in anaphase and the chromatin bridges in telophase and cytokinesis (Figure 6.7a and c). The number of defects

in the cells where the inhibitor was removed increased ~2 fold for metaphase and anaphase, ~1,5 fold for telophase and cytokinesis compared to the cells maintained in the presence of the inhibitor (Figure 6.7c). The experiments were performed thrice and ~150 cells were counted per phase for each condition, the lower incidence of telophase and cytokinesis defects could be attributed to a delay to proceed to the final phase of mitosis due to defects earlier on in mitosis or to cells that have entered mitosis before total cleavage of the NH_2-terminus was achieved.

Taken collectively these data show that the Hara-kiri approach allows us to address the specific questions at hand and that the CENP-A NH_2-terminus is required for proper mitosis.

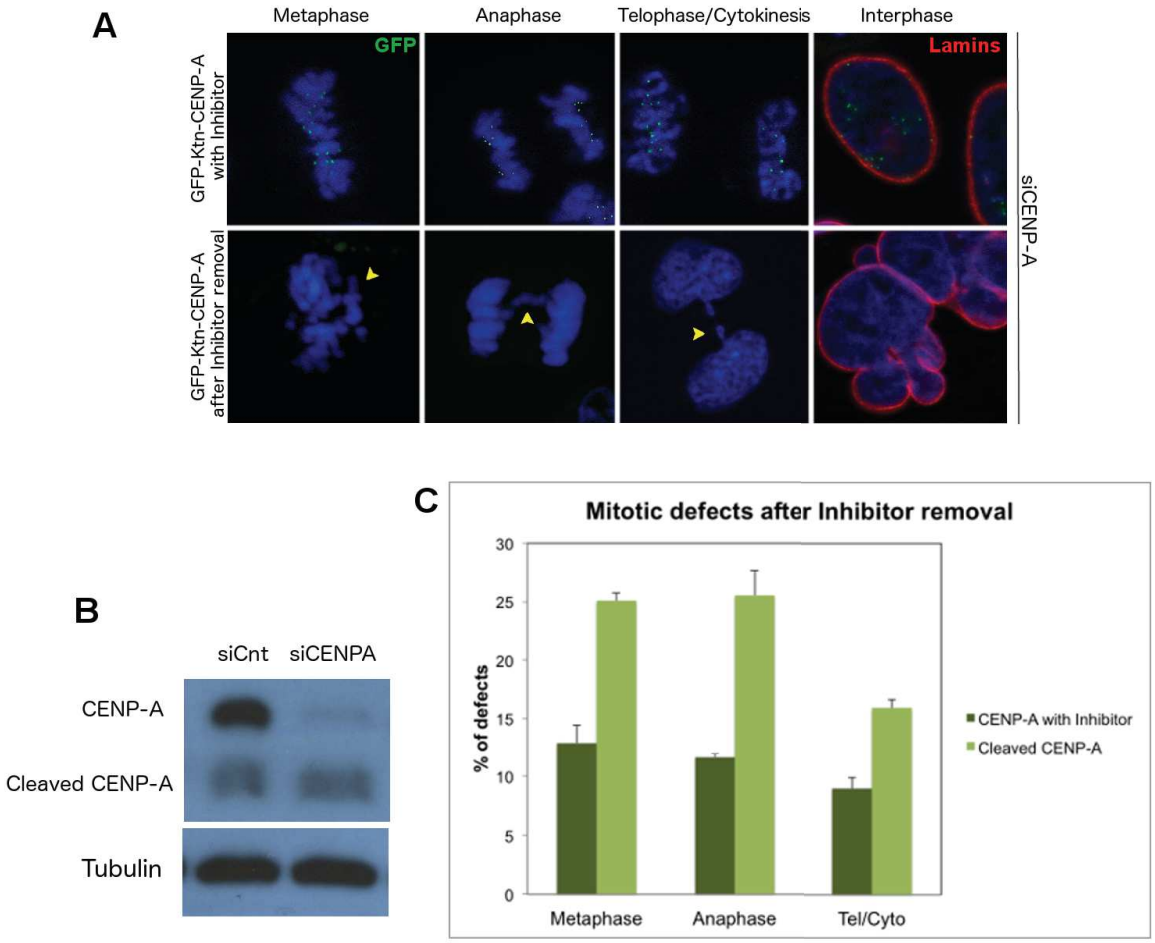


Figure 6.7: The utilization of the Hara-kiri approach permits the cleavage of the CENP-A amino-terminus, which results in increased mitotic defects. A) The cleavage of the GFP-Katana-CENP-A fusion at the onset of mitosis leads to mitotic defects in HeLa cells; highlighted by yellow arrowheads in second row. B) Western blot showing siRNA mediated knock-down of endogenous CENP-A. C) Percentage of mitotic defects observed by phase in cells left in the presence of the inhibitor (dark green columns) and in cells where the inhibitor was removed prior to onset of mitosis (light green columns). Error bars represent standard error of the mean.

PART IV

7. Discussion

7.1. A closer look into CENP-A depletion

One of the significant challenges while studying CENP-A is that it is such an essential protein. Thus eliminating it entirely is very difficult not only because the absence of CENP-A is lethal for cells and organisms but also since 1% of the original CENP-A level is sufficient to maintain at least partial centromere function and to nucleate the assembly of most kinetochore proteins (Fachinetti et al., 2013). *S. cerevisiea* has only one Cse4 containing nucleosome per centromere (Henikoff et al., 2012a) while in humans it is estimated to be around 100 CENP-A nucleosomes. Centromeric chromatin is not composed entirely of CENP-A containing nucleosomes but is interspaced with H3 nucleosomes where approximately one in eight centromeric nucleosomes contain CENP-A (Bodor et al., 2014). Hence the amount of CENP-A present within the centromeric chromatin is significantly greater than the minimum amount required to ensure function of the centromere as the protein is such an essential one.

Thus it is difficult to assess the full impact of CENP-A depletion. In this work we have used two separate ways to deplete CENP-A in cells to analyze the role of the amino-tail of CENP-A and its phosphorylation in mitosis. We established conditional homozygous knockout/knockin (cKO/KI) mouse cell lines. Using these mice we derived mouse embryonic fibroblasts (MEF), which, after immortalization, were infected with constructs expressing a tamoxifen inducible Cre-ERT2 recombinase or with AdenoCRE recombinase. Treatment with tamoxifen of these cells resulted in excision of the two CENP-A alleles and depletion of essentially all protein 6 days after tamoxifen treatment and in 4 days when directly infected with AdenoCRE. Direct infection with AdenoCRE recombinase proved to be quicker and more effective. The tamoxifen induced CENP-A depletion resulted in a small population of cells where the Cre-ERT2 recombinase was not expressed, probably through silencing and theses cells exhibited a growth advantage over the population of cells where CENP-A depletion was successful. This resulted in the appearance of a persistence band on the western blots in knock-out cells as well as in cells infected with the non-phosphorylatable GFP-H3SA-CENP-A construct. However this band was absent in cells infected with GFP-CENP-A and GFP-H3-CENP-A where the knock-out phenotype was rescued. Since AdenoCRE infection proved more effective the rest of the study was conducted using this method.

In the second part of our study, we applied the Hara-kiri approach in HeLa cells and depleted CENP-A levels using siRNA. However, knock-down approaches are less than ideal method as the effect is temporary, especially for proteins such as CENP-A with long half-lives. However, RNA interference directed against the CENP-A mRNA made it possible to obtain a drastic reduction in the expression of the endogenous form of CENP-A and was sufficient to document increased mitotic defects upon cleavage of the CENP-A amino-terminus. This is perhaps also due to the fact that the cells were preincubated with the inhibitor for three days and the presence of endogenous CENP-A at centromeres was already diluted by the presence of the hara-kiri fusion CENP-A prior to siRNA treatment. In fact, it is of great interest to us to set up the hara-kiri approach in our MEF cell model and we accept to see a higher fold increase in defects caused by the cleavage of the CENP-A amino-terminus. Primarily because it is a better model to fully and permanently deplete endogenous CENP-A and additionally because a diploid cell model is preferable to study mitotic defects.

7.2. The importance of the amino-terminal domain of CENP-A and its phosphorylation

The mitotic phosphorylation of CENP-A tail at serine 7 was reported only in human cells (Zeitlin et al., 2001) and is an important event required for proper chromosome segregation and mitotic progression (Goutte-Gattat et al., 2013). This suggests that mitotic phosphorylation of the CENP-A tail should be observed in other metazoans and that it could have a role similar to that in human cells.

Since no specific antibodies against mouse CENP-A and its potentially phosphorylated mitotic form(s) existed, we have used an indirect approach. We first showed that in MEF cells expressing a chimeric CENP-A mutant where the amino-tail was swapped with that of histone H3 (GFP-H3-CENP-A) was able to rescue the mitotic phenotype of MEFs depleted of endogenous CENP-A. However, this was not the case in MEF cells that were expressing the non-phosphorylatable swapped mutant GFP-H3SA-CENP-A, as was the case in HeLa cells (Goutte-Gattat et al., 2013). Since the tail of H3 is distinct in both length and sequence from that of CENP-A, these results strongly suggest that the mitotic function of the NH_2-terminal domain of CENP-A lies entirely in its phosphorylation and not in its length nor in its primary amino-acid sequence. Furthermore we found that the non-phosphorylatable GFP-SA-CENP-A mutant behaved as the non-phosphorytable swapped mutant GFP-H3SA-CENP-A and was unable to rescue the

mitotic phenotype of cells depleted of CENP-A. Our data further identified among the seven serines present in the CENP-A tail, the first three of them, namely S15, S16 and S22, as potential sites for phosphorylation, since the mutant GFP-S15.16.22A (where these three serines were substituted with alanines) behaved as the completely non-phosphorylatable GFP-SA-CENP-A mutant and has lost the ability to rescue the mitotic defects of MEFs depleted of CENP-A.

In brief, our data reveals that the mouse CENP-A, as human CENP-A, appears to be phosphorylated during mitosis and its phosphorylation plays an essential function in chromosome segregation. This result is also consistent with previous study showing that in *S. cerevisiae*, expression of a non-phosphorylatable form of Cse4p leads to a 2 to 3 fold increase in the rate of chromosome segregation defects (Chen et al., 2000).

Other studies do not focus on the phosphorylation of the amino-tail but rather the tail itself and bare a general consensus that the amino-terminal domain is important for centromere function but the details of this function have been conflicting. More recent work has proposed diverse functions for the amino-tail using rescue experiments with chimeric protein mutants. In human diploid cells it was shown that kinetochore assembly requires along with the CATD domain of CENP-A either the amino- or the carboxy-terminal tail. The same study found that in fission yeast the CATD and the amino-terminal domain of Cnp1 were both necessary for centromere function (Fachinetti et al., 2013). Additionally in a separate study, Cnp1 amino-terminus mutants were shown to be viable but exhibited elevated levels of chromosome loss and centromere inactivation (Folco et al., 2015). A study in U2OS cells revealed that in early stages of centromere establishment both tails of CENP-A were required along with the CATD domain but that they were not necessary for centromere maintenance (Logsdon et al., 2015).

These results may seem initially conflicting with our results but a closer look reveals that this is not the case. Already in *S. pombe* there is agreement that the amino-tail is essential for centromeric function. However in human cells the amino tail doesn't seem to be indispensible for centromere function either if the carboxy-tail is present or if the centromere has already been established (Fachinetti et al., 2013; Logsdon et al., 2015). But it is important to notice that these studies were done using H3 chimeras, so in both studies the CENP-A replacements do in fact still have phophorylatable residues at the amino tails.

Hence if the mitotic function of CENP-A amino-tail is dependent on phosphorylation, it will not have been noticed in these studies. Moreover, results we have obtained using the

hara-kiri approach clearly show direct involvement of the amino-tail during mitosis as cleavage of the GFP-Katana-CENP-A fusion at the onset of mitosis leads to increased mitotic defects in cells depleted of endogenous CENP-A. Thus, centromeric establishment and participation in mitosis are two distinct functions of CENP-A amino-terminal domain in humans.

Strikingly in *S. pombe* H3 chimeras were not sufficient to assure centromeric function (Fachinetti et al., 2013) and Cpn1 amino-tail deletion mutants exhibited similar phenotype as the H3 tail swapped Cpn1 mutant (Folco et al., 2015). Indicating that, unlike in *S. cerevisiae*, Cpn1 is probably not phosphorylated. In fact Cpn1 amino-tail has only a single serine at S5 contrary to the serine rich amino-tail of Cse4.

7.3. The phosphorylation of CENP-A and CENP-C involvement

We have established the mitotic function of the N-terminal domain of CENP-A and its phosphorylation, it remains to elucidate what is the function of this phosphorylation. As mentioned before, in humans CENP-A is phosphorylated on serine 7 during prophase and prometaphase (Zeitlin et al., 2001b). Phosphorylation is transient and is no longer detectable from telophase on. It appears to be mediated by Aurora A in prophase and Aurora B in prometaphase (Kunitoku et al., 2003). It is necessary for the proper chromosome congression and segregation as well as for the completion of cytokinesis (Zeitlin et al., 2001a).

In HeLa cells mitotic phosphorylation of human CENP-A is required for kinetochore function (Goutte-Gattat et al., 2013) as the absence of amino-tail phosphorylation resulted in an increase chromosome segregation defects and partial loss of CENP-C from the centromeres. We show that in MEFs, as in HeLa cells the centromeric localization of CENP-C is partially lost in the absence of CENP-A amino-tail phosphorylation. The expression of either GFP-S15.16.22A-CENP-A or GFP-SA-CENP-A in CENP-A depleted MEF's does not lead, in contrast to the phosphorytable counterparts, to rescue of the centromere localization of CENP-C. This was also the case in H3 tail swap mutants where GFP-H3SA-CENP-A expression resulted in partial CENP-C loss at centromeres upon CENP-A depletion while GFP-H3-CENP-A cells showed no change in CENP-C localization. Therefore the recruitment or maintenance of CENP-C on the centromeric chromatin depends on the phosphorylation of the NH_2-terminal domain of CENP-A and it is likely that this is the essential mitotic function of the NH_2-terminal domain of CENP-A.

So it should be asked how can the phosphorylation of CENP-A participate in the centromeric localization of CENP-C? Is it through a direct or indirect interaction with the amino-tail? It should be noted that at present there is no evidence of direct interaction between the amino-tail of CENP-A and CENP-C. The literature suggests that the interaction between the two proteins occurs at the carboxy-terminal end of CENP-A while the central region of CENP-C directly binds to the CENP-A nucleosome (Carroll et al., 2010; Kato et al., 2013). Another hypothesis, which we favor is indirect interaction and supposes the existence of an adaptive protein linking CENP-A and CENP-C. Such a protein remains to be identified, but the proteins of the 14-3-3 family seem to be good candidates. These proteins specifically bind phosphorylated units and serve as adapters in a large number of processes (for review, see Hermeking and Benzinger, 2006). We hypothesize that one of these proteins associates with the phosphorylated NH_2-terminal extension of CENP-A and interacts simultaneously with CENP-C, thus stabilizing its attachment to centromeric chromatin as in the case of HeLa cells (Figure 7.1).

Against this hypothesis, it should be noted that the peptide sequence of CENP-A in the vicinity of serine 7 does not correspond to any of the two consensus motifs recognized by the 14-3-3 (RSXSXP or RXXXSXP); However, other proteins are known to interact with 14-3-3 without meeting these consensus (Aitken, 2006). Furthermore, it has recently been shown that 14-3-3 interact with the NH_2-terminal extension of H3 on its phosphorylated residues S10 and S28 (Winter et al., 2008). This could explain the efficacy of the amino-terminal tail of H3 to replace that of CENP-A in cells as well at the presence of serine residues in the tails of the CENP-A of different species located at different positions. We speculate that the first serine(s) within the tails of the CENP-A proteins in metazoan would be, due to their higher accessibility, the ones phosphorylated at the onset of mitosis.

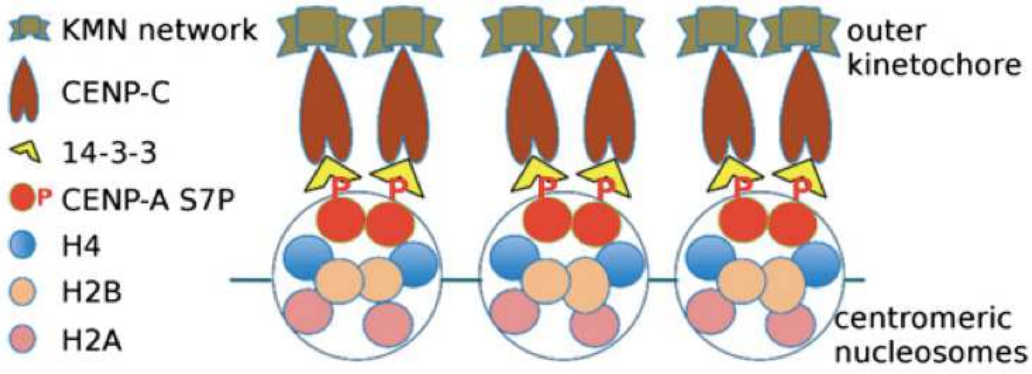


Figure 7.1 Schematics describing how phosphorylated CENP-A and CENP-C can act with 14-3-3 proteins in the assembly and maintenance of active kinetochores. (adopted from Goutte-Gattat et al, 2013)

7.4. Mitotic function, centromere establishment or maintenance?

In this study we have showed that the phosphorylation of the amino-terminal domain of CENP-A is important for proper mitosis and CENP-C localization in MEF cells. We also showed that the presence of amino-terminal domain of CENP-A is necessary during mitosis for proper chromosome segregation in HeLa cells. Therefore the recruitment or maintenance of CENP-C on centromeric chromatin depends on the phosphorylation of the NH_2-terminal domain of CENP-A and it is likely that this is the essential mitotic function of the amino-terminal domain of CENP-A.

Defects in chromosome segregation often stir the focus towards the spindle assembly checkpoint (SAC), which precisely serves to prevent such defects. In humans, CENP-A phosphorylation is limited to mitosis and is no longer detectable from telophase on and CENP-C (Kunitoku et al., 2003) was previously reported to be involved in chromosome segregation and mitotic checkpoint function (Kwon et al., 2007). Thus, CENP-C might provide an attachment site for the recruitment of outer kinetochore components and their associated partners, including the components of the spindle assembly checkpoint (Screpanti et al., 2011). It has been reported that the Mis12 complex interacts directly with the inner kinetochore via CENP-C. When the interaction between CENP-C and Mis12 is disrupted cells experience checkpoint impairment.

A more recent study has shown that CENP-C interactions are dynamic and cell cycle–dependent. They suggest that CENP-C is bound weaker to CENP-A nucleosomes during interphase through different interactions then in contrast to mitosis where CENP-C binds to CENP-A nucleosomes via its C-terminal dimerization domain (Nagpal et al., 2015). Considering the above mentioned information, it can be speculated that stronger CENP-C binding during mitosis is important for chromosome segregation. This would not be surprising since centromeres go through increased tension and pulling as a result of microtubule attachment, in fact a loss of spindle tension between the two kinetochores of the same chromosome trigger SAC activation since it is a sign of incorrect MT attachments. In the absence of sufficient tension, the Aurora B kinase that is localized at the centromeres during metaphase, destabilizes the interactions between the kinetochore and microtubules. Aurora B may act by directly phosphorylating members KMN network, reducing their affinity for microtubules (Welburn et al., 2010).

Up to now we have concentrated on the mitotic function of the amino-terminal domain of CENP-A. Though our study has linked the amino-tail function directly to

mitosis by using the hara-kiri approach, it doesn't exclude the amino-tail from having extra-mitotic functions. In fact there has been several reports that link CENP-A amino-tail function to the recruitment of other CCAN proteins as well as that of CENP-C. A study in human diploid cells has shown that the NH_2-terminal domain of CENP-A is important to stabilize CENP-B (but no other CCAN proteins) for kinetochore maintenance (Fachinetti et al., 2013). In contrast, a separate study in U2OS cells revealed that the amino-tail was essential in early stages of centromere establishment in a CENP-B independent manner. The study linked amino-tail function to indirect stabilization of CENP-C binding and to directly or indirectly promoting CENP-T recruitment. However, the same study found that the amino-tail was not essential for centromere maintenance (Logsdon et al., 2015). Studies in *S. pombe* found that Cnp1 amino-terminus mutants were shown to be viable but exhibited elevated levels of chromosome loss and centromere inactivation. Amino-terminus mutants had reduced levels of CENP-T branch of the CCAN but not that of CENP-C, indicating a role for Cpn1 amino-tail in centromere maintenance (Folco et al., 2015).

In a recent review by Drinnenberg and colleagues, the auteurs compare the kinetochore to the ship of Theseus, asking whether the ship was still the same ship if one has changed all its planks, highlighting the recurrent evolutionary turnover of kinetochore proteins. Though the protein composition of kinetochores is similar across species as divergent as yeast to humans, recent findings have revealed an unexpected degree of compositional diversity in kinetochores as some proteins that are essential in some species have been lost in others or have functionally diverged to acquire either essential or redundant roles (Drinnenberg et al., 2016).

Additionally, trying to assess the order of molecular events down-stream of CENP-A is very challenging experimentally partially due the low abundance of CENP-A nucleosomes compared to H3 nucleosomes, making biochemical analysis more challenging and largely due to the slow turnover of CENP-A molecules in the cell. Even when CENP-A is depleted using AdenoCRE recombinase based methods, it is hard to asses in a mixed population of cells the exact moment of total CENP-A depletion coupled with rapid decline in proliferation and increase in mitotic defects. So especially when assessing centromere establishment or immediate molecular effects, new models are needed. This is why we have devised the hara-kiri approach.

7.5. Perspectives

Perspectives regarding the phosphorylation of the NH_2-terminus of mouse CENP-A in MEF cells: The first step forward in this project will be to pin point the serine or serines that are phosphorylated in the mouse CENP-A tail. It would be also interesting to evaluate whether the localization of other CCAN proteins, mainly CENP-T and CENP-B are affected by amino-tail phosphorylation and to quantify the effects on these three proteins. What is the extent of kinetochore disruption in the absence of CENP-A phosphorylation and what are the downstream effects of partial CENP-C dislocation?

Additionally, we would like to show that the 14-3-3 proteins are the "bridges" involved in the interaction between CENP-C and the phosphorylated N-terminal domain of CENP-A, as in HeLa cells.

Eventually it would be desirable to elucidate the kinase responsible for the phosphorylation of the amino-tail. Are CENP-A homologues in other eukaryotes also phosphorylated? Does it have the same effect on CENP-C?

Perspectives regarding the functions of the NH_2-terminus and the use of the Hara-kiri approach in HeLa cells: The extent of experiments we were able to run with the Hara-kiri approach was limited due to the very limited supply of the inhibitor we had. Fortunately, the inhibitor has recently been commercialized and we can now purchase it. We would like to initially perform the cleavage experiments and reproduce results using live microscopy. This will also provide us with additional information on whether the length of mitosis is prolonged due to the absence of the amino-tail.

It would be also interesting to evaluate whether the localization of CCAN proteins, mainly CENP-C but also CENP-T and CENP-B after amino-tail cleavage with the Hara-kiri method. We would like to pursue the localization of these proteins right after NH_2-terminus cleavage but also at the upcoming mitosis after the cleavage.

As mentioned before, the Hara-kiri approach is a new approach we have devised and is applicable to other proteins. It can be used in a similar way to our project to study functions of other histone tails in time or to study the function other proteins implicated in rapid phased processes like mitosis. We expect this approach to become a valuable tool in future research. Eventually we would like to use this approach to also further study the function of the carboxy-tail of CENP-A, with special interest to its role in CENP-C stabilization and centromere maintenance.

A new drug inducible method for controllable CENP-A degradation: A plant auxin-inducible degron (AID) system has been shown to degrade AID-tagged target proteins in nonplant cells (Nishimura et al., 2009). This method has recently become available and much like the Hara-kiri approach can be a very useful tool to study CENP-A function. The AID-tagged CENP-A can be used to replace in MEF cells the endogenous CENP-A that we can deplete using AdenoCRE infection. These cells in turn can be invaluable since CENP-A null phenotype can be induced rapidly be after auxin addition. In fact, Cleaveland lab has shown that induced CENP-A degradation occurs rapidly after addition of auxin with protein half-life reduced to as little as 9 min. Additionally, AID-mediated degradation is demonstrated to be rapidly reversible upon auxin removal (Holland et al., 2012). It can be very interesting to combine the two approaches to the study of CENP-A tails.

ANNEXES

8. Other publications

Paper has been accepted, publication pending in Biochemsitry and Cell Biology.

Histone H3.3 regulates mitotic progression in mouse embryonic fibroblasts

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Abstract

H3.3 is a histone variant, which marks transcription start sites as well as telomeres and heterochromatic sites on the genome. H3.3 presence is thought to positively correlate with transcriptional status of its target genes. Using a conditional genetic strategy against H3.3B combined with short hairpin RNAs against H3.3A, we essentially depleted all H3.3 gene expression in mouse embryonic fibroblasts. Following nearly complete loss of H3.3 in cells, our transcriptomic analyses show very little impact on global gene expression as well as on histone variant H2A.Z localization. Instead, fibroblasts display slower cell growth and an increase in cell death coincident with large-scale chromosome misalignment in mitosis and large polylobed or micronuclei in interphase cells. Thus we conclude that H3.3 may additionally have an important under-explored role in chromosome segregation, nuclear structure and maintenance of genome integrity.

Keywords

H3.3, transcription, Mouse embryonic fibroblasts, RNA-seq, mitosis

Introduction

The replacement of canonical histones, core constituents of chromatin, by their variants drives chromatin dynamics and allows for functional and structural regulation of key cellular mechanisms (Boulard et al. 2007, Henikoff 2008). In contrast with replication dependent (RD) histones, the deposition of replication independent (RI) occurs in a cell cycle independent manner (Henikoff et al. 2004). There are RI variants for all the RD histones except for histone H4 (Hake et al. 2005, Pusarla and Bhargava 2005). The histone H3 variant H3.3 differs from its RD counterparts H3.1 and H3.2 by

9. Bibliography

Abbott, D. W., V. S. Ivanova, X. Wang, W. M. Bonner, and J. Ausió (2001). Characterization of the stability and folding of H2A.Z chromatin particles: implications for transcriptional activation. *J. Biol. Chem.* **2001,** 276:41945–41949.

Adams, R. R.; Carmena, M.; Earnshaw, W. C., Chromosomal passengers and the (aurora) ABCs of mitosis. *Trends Cell Biol* **2001,** *11* (2), 49-54.

Afonso, O.; Matos, I.; Maiato, H., Spatial control of the anaphase-telophase transition. *Cell Cycle* **2014,** *13* (19), 2985-6.

Ahmad, K. and S. Henikoff (2002). The histone variant H3.3 marks active chromatin by replication-independent nucleosome assembly. *Mol. Cell* **2002,** 9:1191–1200.

Aitken, A. (2006). 14-3-3 proteins: a historic overview. *Semin. Cancer Biol.* **2006,** 16:162–172.

Allis, C. D.; Berger, S. L.; Cote, J.; Dent, S.; Jenuwien, T.; Kouzarides, T.; Pillus, L.; Reinberg, D.; Shi, Y.; Shiekhattar, R.; Shilatifard, A.; Workman, J.; Zhang, Y., New nomenclature for chromatin-modifying enzymes. *Cell* **2007,** *131* (4), 633-6.

Amato, A., T. Schillaci, L. Lentini, and A. Di Leonardo (2009). CENPA overexpression promotes genome instability in pRb-depleted human cells. *Mol. Cancer* **2009,** 8:119.

Angelov, D., A. Molla, P.-Y. Perche, F. Hans, J. Côté, S. Khochbin, P. Bouvet, and S. Dimitrov (2003). The histone variant macroH2A interferes with transcription factor binding and SWI/SNF nucleosome remodeling. *Mol. Cell* **2003,** 11:1033–1041.

Arents, G. and E. N. Moudrianakis (1995). The histone fold: a ubiquitous architectural motif utilized in DNA compaction and protein dimerization. *Proc. Natl. Acad. Sci. USA* **1995**, 92:11170–11174.

Arimura, Y.; Kimura, H.; Oda, T.; Sato, K.; Osakabe, A.; Tachiwana, H.; Sato, Y.; Kinugasa, Y.; Bahe, S., Y.-D. Stierhof, C. J. Wilkinson, F. Leiss, and E. A. Nigg (2005). Rootletin forms centriole-associated filaments and functions in centrosome cohesion. *J. Cell Biol.*, **171**:27–33.

Banerjee, T.; Chakravarti, D., A peek into the complex realm of histone phosphorylation. *Mol Cell Biol* **2011,** *31* (24), 4858-73.

Bannister, A. J., P. Zegerman, J. F. Partridge, E. A. Miska, J. O. Thomas, R. C. Allshire, and T. Kouzarides (2001). Selective recognition of methylated lysine 9 on histone H3 by the HP1 chromo domain. *Nature*, **410**:120–124.

Bao, Y. and X. Shen (2007). INO80 subfamily of chromatin remodeling complexes. *Mutat. Res.*, **618**:18–29.

Bao, Y., K. Konesky, Y.-J. Park, S. Rosu, P. N. Dyer, D. Rangasamy, D. J. Tremethick, P. J. Laybourn, and K. Luger (2004). Nucleosomes containing the histone variant H2A.Bbd

organize only 118 base pairs of DNA. *EMBO J*., **23**:3314–3324.

Bednar, J.; Hamiche, A.; Dimitrov, S., H1-nucleosome interactions and their functional implications. *Biochim Biophys Acta* **2016,** *1859* (3), 436-43.

Belmont, A. S., J. W. Sedat, and D. A. Agard (1987). A three-dimensional approach to mitotic chromosome structure: evidence for a complex hierarchical organization. *J. Cell Biol*., **105**:77–92.

Biermann, K., L. C. Heukamp, K. Steger, H. Zhou, F. E. Franke, I. Guetgemann, V. Sonnack, R. Brehm, J. Berg, and P. J. Bastian (2007). Gene expression profiling identifies new biological markers of neoplastic germ cells. *Anticancer Res*., **27**:3091– 3100.

Black, B. E., D. R. Foltz, S. Chakravarthy, K. Luger, V. L. Woods, and D. W. Cleve- land (2004). Structural determinants for generating centromeric chromatin. *Nature*, **430**:578–582.

Black, B. E., M. A. Brock, S. Bédard, V. L. Woods, and D. W. Cleveland (2007a). An epigenetic mark generated by the incorporation of CENP-A into centromeric nucleosomes. *Proc. Natl. Acad. Sci. USA*, **104**:5008–5013.

Black, B. E.; Bassett, E. A., The histone variant CENP-A and centromere specification. *Curr Opin Cell Biol* **2008,** *20* (1), 91-100.

Blower, M. D. and G. H. Karpen (2001). The role of Drosophila CID in kinetochore formation, cell-cycle progression and heterochromatin interactions. *Nat. Cell Biol*., **3**:730–739.

Blower, M. D., B. A. Sullivan, and G. H. Karpen (2002). Conserved organization of centromeric chromatin in flies and humans. *Dev. Cell*, **2**:319–330.

Bodor, D. L.; Mata, J. F.; Sergeev, M.; David, A. F.; Salimian, K. J.; Panchenko, T.; Cleveland, D. W.; Black, B. E.; Shah, J. V.; Jansen, L. E., The quantitative architecture of centromeric chromatin. *Elife* **2014,** *3*, e02137.

Botuyan, M. V.; Lee, J.; Ward, I. M.; Kim, J. E.; Thompson, J. R.; Chen, J.; Mer, G., Structural basis for the methylation state-specific recognition of histone H4-K20 by 53BP1 and Crb2 in DNA repair. *Cell* **2006,** *127* (7), 1361-73.

Bouchet-Marquis, C.; Dubochet, J.; Fakan, S., Cryoelectron microscopy of vitrified sections: a new challenge for the analysis of functional nuclear architecture. *Histochem Cell Biol* **2006,** *125* (1-2), 43-51.

Boulard, M., T. Gautier, G. O. Mbele, V. Gerson, A. Hamiche, D. Angelov, P. Bouvet, and S. Dimitrov (2006). The NH2 tail of the novel histone variant H2BFWT exhibits properties distinct from conventional H2B with respect to the assembly of mitotic chromosomes. *Mol. Cell. Biol*., **26**:1518–1526.

Bouzinba-Segard, H., A. Guais, and C. Francastel (2006). Accumulation of small murine

minor satellite transcripts leads to impaired centromeric architecture and function. *Proc. Natl. Acad. Sci. USA*, **103**:8709–8714.

Boy de la Tour, E.; Laemmli, U. K., The metaphase scaffold is helically folded: sister chromatids have predominantly opposite helical handedness. *Cell* **1988,** *55* (6), 937-44.

Boyarchuk, E.; Filipescu, D.; Vassias, I.; Cantaloube, S.; Almouzni, G., The histone variant composition of centromeres is controlled by the pericentric heterochromatin state during the cell cycle. *J Cell Sci* **2014,** *127* (Pt 15), 3347-59.

Brittle, A. L. and H. Ohkura (2005). Centrosome maturation: Aurora lights the way to the poles. *Curr. Biol.*, **15**:R880–R882.

Bruce, K.; Myers, F. A.; Mantouvalou, E.; Lefevre, P.; Greaves, I.; Bonifer, C.; Tremethick, D. J.; Thorne, A. W.; Crane-Robinson, C., The replacement histone H2A.Z in a hyperacetylated form is a feature of active genes in the chicken. *Nucleic Acids Res* **2005,** *33* (17), 5633-9.

Brust-Mascher, I. and J. M. Scholey (2002). Microtubule flux and sliding in mitotic spindles of Drosophila embryos. *Mol. Biol. Cell*, **13**:3967–3975.

Brust-Mascher, I., G. Civelekoglu-Scholey, M. Kwon, A. Mogilner, and J. M. Scholey (2004). Model for anaphase B: role of three mitotic motors in a switch from poleward flux to spindle elongation. *Proc. Natl. Acad. Sci. U.S.A.*, **101**:15938–15943.

Bush, K. M.; Yuen, B. T.; Barrilleaux, B. L.; Riggs, J. W.; O'Geen, H.; Cotterman, R. F.; Knoepfler, P. S., Endogenous mammalian histone H3.3 exhibits chromatin-related functions during development. *Epigenetics Chromatin* **2013,** *6* (1), 7.

Bustin, M.; Catez, F.; Lim, J. H., The dynamics of histone H1 function in chromatin. *Mol Cell* **2005,** *17* (5), 617-20.

Buvelot, S., S. Y. Tatsutani, D. Vermaak, and S. Biggins (2003). The budding yeast Ipl1/Aurora protein kinase regulates mitotic spindle disassembly. *J. Cell Biol.*, **160**:329–339.

Carroll, C. W., K. J. Milks, and A. F. Straight (2010). Dual recognition of CENP-A nucleosomes is required for centromere assembly. *J. Cell Biol.*, **189**:1143–1155.

Carroll, C. W., M. C. C. Silva, K. M. Godek, L. E. T. Jansen, and A. F. Straight (2009). Centromere assembly requires the direct recognition of CENP-A nucleosomes by CENP-N. *Nat. Cell Biol.*, **11**:896–902.

Chadwick, B. P. and H. F. Willard (2001). A novel chromatin protein, distantly related to histone H2A, is largely excluded from the inactive X chromosome. *J. Cell Biol.*, **152**:375–384.

Chakravarthy, S.; Gundimella, S. K.; Caron, C.; Perche, P. Y.; Pehrson, J. R.; Khochbin, S.; Luger, K., Structural characterization of the histone variant macroH2A. *Mol Cell Biol* **2005,** *25* (17), 7616-24.

Chan, E. H. Y., A. Santamaria, H. H. W. Silljé, and E. A. Nigg (2008). Plk1 regu- lates mitotic Aurora A function through betaTrCP-dependent degradation of hBora. *Chromosoma*, **117**:457–469.

Chao, W. C.; Kulkarni, K.; Zhang, Z.; Kong, E. H.; Barford, D., Structure of the mitotic checkpoint complex. *Nature* **2012,** *484* (7393), 208-13.

Cheeseman, I. M. and A. Desai (2008). Molecular architecture of the kinetochore-microtubule interface. *Nat. Rev. Mol. Cell Biol.*, **9**:33–46.

Cheeseman, I. M., J. S. Chappie, E. M. Wilson-Kubalek, and A. Desai (2006). The conserved KMN network constitutes the core microtubule-binding site of the kinetochore. *Cell*, **127**:983–997.

Chen, C. C.; Dechassa, M. L.; Bettini, E.; Ledoux, M. B.; Belisario, C.; Heun, P.; Luger, K.; Mellone, B. G., CAL1 is the Drosophila CENP-A assembly factor. *J Cell Biol* **2014,** *204* (3), 313-29.

Chen, Y., R. E. Baker, K. C. Keith, K. Harris, S. Stoler, and M. Fitzgerald-Hayes (2000). The N terminus of the centromere H3-like protein Cse4p performs an essential function distinct from that of the histone fold domain. *Mol. Cell. Biol.*, **20**:7037– 7048.

Chen, Y.; Riley, D. J.; Zheng, L.; Chen, P. L.; Lee, W. H., Phosphorylation of the mitotic regulator protein Hec1 by Nek2 kinase is essential for faithful chromosome segregation. *J Biol Chem* **2002,** *277* (51), 49408-16.

Churikov, D., J. Siino, M. Svetlova, K. Zhang, A. Gineitis, E. Morton Bradbury, and A. Zalensky (2004). Novel human testis-specific histone H2B encoded by the interrupted gene on the X chromosome. *Genomics*, **84**:745–756.

Ciferri, C., A. Musacchio, and A. Petrovic (2007). The Ndc80 complex: hub of kineto-chore activity. *FEBS Lett.*, **581**:2862–2869.

Cimini, D., X. Wan, C. B. Hirel, and E. D. Salmon (2006). Aurora kinase promotes turnover of kinetochore microtubules to reduce chromosome segregation errors. *Curr. Biol.*, **16**:1711–1718.

Clapier, C. R.; Cairns, B. R., The biology of chromatin remodeling complexes. *Annu Rev Biochem* **2009,** *78*, 273-304.

Cloos, P. A.; Christensen, J.; Agger, K.; Helin, K., Erasing the methyl mark: histone demethylases at the center of cellular differentiation and disease. *Genes Dev* **2008,** *22* (9), 1115-40.

Corona, D. F. V. and J. W. Tamkun (2004). Multiple roles for ISWI in transcription, chromosome organization and DNA replication. *Biochim. Biophys. Acta*, **1677**:113– 119.

Cosgrove, M. S.; Boeke, J. D.; Wolberger, C., Regulated nucleosome mobility and the histone code. *Nat Struct Mol Biol* **2004,** *11* (11), 1037-43.

Couldrey, C.; Carlton, M. B.; Nolan, P. M.; Colledge, W. H.; Evans, M. J., A retroviral gene trap insertion into the histone 3.3A gene causes partial neonatal lethality, stunted growth, neuromuscular deficits and male sub-fertility in transgenic mice. *Hum Mol Genet* **1999,** *8* (13), 2489-95.

Cremer, T.; Cremer, M., Chromosome territories. *Cold Spring Harb Perspect Biol* **2010,** *2* (3), a003889.

Daal, A. and S. C. Elgin (1992). A histone variant, H2AvD, is essential in Drosophila melanogaster. *Mol. Biol. Cell*, **3**:593–602.

Dambacher, S.; Deng, W.; Hahn, M.; Sadic, D.; Fröhlich, J.; Nuber, A.; Hoischen, C.; Diekmann, S.; Leonhardt, H.; Schotta, G., CENP-C facilitates the recruitment of M18BP1 to centromeric chromatin. *Nucleus* **2012,** *3* (1), 101-10.

Dekker, J.; Marti-Renom, M. A.; Mirny, L. A., Exploring the three-dimensional organization of genomes: interpreting chromatin interaction data. *Nat Rev Genet* **2013,** *14* (6), 390-403.

Desai, A. and T. J. Mitchison (1997). Microtubule polymerization dynamics. *Annu. Rev. Cell Dev. Biol.*, **13**:83–117.

Dhalluin, C., J. E. Carlson, L. Zeng, C. He, A. K. Aggarwal, and M. M. Zhou (1999). Structure and ligand of a histone acetyltransferase bromodomain. *Nature*, **399**:491– 496.

Dixon, J. R.; Selvaraj, S.; Yue, F.; Kim, A.; Li, Y.; Shen, Y.; Hu, M.; Liu, J. S.; Ren, B., Topological domains in mammalian genomes identified by analysis of chromatin interactions. *Nature* **2012,** *485* (7398), 376-80.

Dorigo, B., T. Schalch, A. Kulangara, S. Duda, R. R. Schroeder, and T. J. Richmond (2004). Nucleosome arrays reveal the two-start organization of the chromatin fiber. *Science*, **306**:1571–1573.

Downs, J. A.; Lowndes, N. F.; Jackson, S. P., A role for Saccharomyces cerevisiae histone H2A in DNA repair. *Nature* **2000,** *408* (6815), 1001-4.

Doxsey, S., W. Zimmerman, and K. Mikule (2005). Centrosome control of the cell cycle. *Trends Cell Biol.*, **15**:303–311.

Doyen, C.-M., F. Montel, T. Gautier, H. Menoni, C. Claudet, M. Delacour-Larose, D. Angelov, A. Hamiche, J. Bednar, and C. Faivre-Moskalenko (2006a). Dissection of the unusual structural and functional properties of the variant H2A.Bbd nucleosome. *EMBO J.*, **25**:4234–4244.

Doyen, C.-M., W. An, D. Angelov, V. Bondarenko, F. Mietton, V. M. Studitsky, A. Hamiche, R. G. Roeder, P. Bouvet, and S. Dimitrov (2006b). Mechanism of polymerase II transcription repression by the histone variant macroH2A. *Mol. Cell. Biol.*, **26**:1156–1164.

Drinnenberg, I. A.; Henikoff, S.; Malik, H. S., Evolutionary Turnover of Kinetochore Proteins: A Ship of Theseus? *Trends Cell Biol* **2016,** *26* (7), 498-510.

Dunleavy, E. M., D. Roche, H. Tagami, N. Lacoste, D. Ray-Gallet, Y. Nakamura, Y. Daigo, Y. Nakatani, and G. Almouzni-Pettinotti (2009). HJURP is a cell- cycle-dependent maintenance and deposition factor of CENP-A at centromeres. *Cell*, **137**:485–497.

Dutertre, S., M. Cazales, M. Quaranta, C. Froment, V. Trabut, C. Dozier, G. Mirey, J.-P. Bouché, N. Theis-Febvre, and E. Schmitt (2004). Phosphorylation of CDC25B by Aurora-A at the centrosome contributes to the G2-M transition. *J. Cell Sci.*, **117**:2523–2531.

Dutertre, S., S. Descamps, and C. Prigent (2002). On the role of aurora-A in centrosome function. *Oncogene*, **21**:6175–6183.

Dutnall, R. N.; Ramakrishnan, V., Twists and turns of the nucleosome: tails without ends. *Structure* **1997,** *5* (10), 1255-9.

Eagen, K. P.; Hartl, T. A.; Kornberg, R. D., Stable Chromosome Condensation Revealed by Chromosome Conformation Capture. *Cell* **2015,** *163* (4), 934-46.

Earnshaw, W. C.; Migeon, B. R., Three related centromere proteins are absent from the inactive centromere of a stable isodicentric chromosome. *Chromosoma* **1985,** *92* (4), 290-6.

Eddé, B.; Rossier, J.; Le Caer, J. P.; Desbruyères, E.; Gros, F.; Denoulet, P., Posttranslational glutamylation of alpha-tubulin. *Science* **1990,** *247* (4938), 83-5.

Eltsov, M.; Maclellan, K. M.; Maeshima, K.; Frangakis, A. S.; Dubochet, J., Analysis of cryo-electron microscopy images does not support the existence of 30-nm chromatin fibers in mitotic chromosomes in situ. *Proc Natl Acad Sci U S A* **2008,** *105* (50), 19732-7.

Faast, R., V. Thonglairoam, T. C. Schulz, J. Beall, J. R. Wells, H. Taylor, K. Matthaei, P. D. Rathjen, D. J. Tremethick, and I. Lyons (2001). Histone variant H2A.Z is required for early mammalian development. *Curr. Biol.*, **11**:1183–1187.

Fachinetti, D.; Folco, H. D.; Nechemia-Arbely, Y.; Valente, L. P.; Nguyen, K.; Wong, A. J.; Zhu, Q.; Holland, A. J.; Desai, A.; Jansen, L. E.; Cleveland, D. W., A two-step mechanism for epigenetic specification of centromere identity and function. *Nat Cell Biol* **2013,** *15* (9), 1056-66.

Falk, S. J.; Guo, L. Y.; Sekulic, N.; Smoak, E. M.; Mani, T.; Logsdon, G. A.; Gupta, K.; Jansen, L. E.; Van Duyne, G. D.; Vinogradov, S. A.; Lampson, M. A.; Black, B. E., Chromosomes. CENP-C reshapes and stabilizes CENP-A nucleosomes at the centromere. *Science* **2015,** *348* (6235), 699-703.

Ferguson, A. M.; White, L. S.; Donovan, P. J.; Piwnica-Worms, H., Normal cell cycle and checkpoint responses in mice and cells lacking Cdc25B and Cdc25C protein phosphatases. *Mol Cell Biol* **2005,** *25* (7), 2853-60.

Fernández-Miranda, G.; Trakala, M.; Martín, J.; Escobar, B.; González, A.; Ghyselinck, N. B.; Ortega, S.; Cañamero, M.; Pérez de Castro, I.; Malumbres, M., Genetic disruption of aurora B uncovers an essential role for aurora C during early mammalian development. *Development* **2011,** *138* (13), 2661-72.

Folco, H. D.; Campbell, C. S.; May, K. M.; Espinoza, C. A.; Oegema, K.; Hardwick, K. G.; Grewal, S. I.; Desai, A., The CENP-A N-tail confers epigenetic stability to centromeres via the CENP-T branch of the CCAN in fission yeast. *Curr Biol* **2015,** *25* (3), 348-56.

Foltz, D. R., L. E. T. Jansen, A. O. Bailey, J. R. Yates, E. A. Bassett, S. Wood, B. E. Black, and D. W. Cleveland (2009). Centromere-specific assembly of CENP-a nucleosomes is mediated by HJURP. *Cell*, **137**:472–484.

Foltz, D. R., L. E. T. Jansen, B. E. Black, A. O. Bailey, J. R. Yates, and D. W. Cleveland (2006). The human CENP-A centromeric nucleosome-associated complex. *Nat. Cell Biol.*, **8**:458–469.

Francis, N. J., R. E. Kingston, and C. L. Woodcock (2004). Chromatin compaction by a polycomb group protein complex. *Science*, **306**:1574–1577.

Fraser, P.; Grosveld, F., Locus control regions, chromatin activation and transcription. *Curr Opin Cell Biol* **1998,** *10* (3), 361-5.

Furuyama, S. and S. Biggins (2007). Centromere identity is specified by a single centromeric nucleosome in budding yeast. *Proc. Natl. Acad. Sci. USA*, **104**:14706– 14711.

Furuyama, T. and S. Henikoff (2009). Centromeric nucleosomes induce positive DNA supercoils. *Cell*, **138**:104–113.

Fussner, E.; Strauss, M.; Djuric, U.; Li, R.; Ahmed, K.; Hart, M.; Ellis, J.; Bazett-Jones, D. P., Open and closed domains in the mouse genome are configured as 10-nm chromatin fibres. *EMBO Rep* **2012,** *13* (11), 992-6.

Gaetz, J. and T. M. Kapoor (2004). Dynein/dynactin regulate metaphase spindle length by targeting depolymerizing activities to spindle poles. *J. Cell Biol.*, **166**:465–471.

Gan, L.; Ladinsky, M. S.; Jensen, G. J., Chromatin in a marine picoeukaryote is a disordered assemblage of nucleosomes. *Chromosoma* **2013,** *122* (5), 377-86.

Ganem, N. J. and D. A. Compton (2004). The KinI kinesin Kif2a is required for bipolar spindle assembly through a functional relationship with MCAK. *J. Cell Biol.*, **166**:473–478.

Gascoigne, K. E., K. Takeuchi, A. Suzuki, T. Hori, T. Fukagawa, and I. M. Cheeseman

(2011a). Induced Ectopic Kinetochore Assembly Bypasses the Requirement for CENP- A Nucleosomes. *Cell*, **145**:410–422.

Gascoigne, K. E.; Cheeseman, I. M., CDK-dependent phosphorylation and nuclear exclusion coordinately control kinetochore assembly state. *J Cell Biol* **2013,** *201* (1), 23-32.

Gautier, T.; Abbott, D. W.; Molla, A.; Verdel, A.; Ausio, J.; Dimitrov, S., Histone variant H2ABbd confers lower stability to the nucleosome. *EMBO Rep* **2004,** *5* (7), 715-20.

Giet, R. and D. M. Glover (2001). Drosophila aurora B kinase is required for histone H3 phosphorylation and condensin recruitment during chromosome condensation and to organize the central spindle during cytokinesis. *J. Cell Biol.*, **152**:669–682.

Giménez-Abián, J. F., I. Sumara, T. Hirota, S. Hauf, D. Gerlich, C. Torre, J. Ellenberg, and J.-M. Peters (2004). Regulation of sister chromatid cohesion between chromosome arms. *Curr. Biol.*, **14**:1187–1193.

Glotzer, M., The 3Ms of central spindle assembly: microtubules, motors and MAPs. *Nat Rev Mol Cell Biol* **2009,** *10* (1), 9-20.

Glover, D. M.; Hagan, I. M.; Tavares, A. A., Polo-like kinases: a team that plays throughout mitosis. *Genes Dev* **1998,** *12* (24), 3777-87.

Golan, A., Y. Yudkovsky, and A. Hershko (2002). The cyclin-ubiquitin ligase activity of cyclosome/APC is jointly activated by protein kinases Cdk1-cyclin B and Plk. *J. Biol. Chem.*, **277**:15552–15557.

Goldberg, A. D.; Banaszynski, L. A.; Noh, K. M.; Lewis, P. W.; Elsaesser, S. J.; Stadler, S.; Dewell, S.; Law, M.; Guo, X.; Li, X.; Wen, D.; Chapgier, A.; DeKelver, R. C.; Miller, J. C.; Lee, Y. L.; Boydston, E. A.; Holmes, M. C.; Gregory, P. D.; Greally, J. M.; Rafii, S.; Yang, C.; Scambler, P. J.; Garrick, D.; Gibbons, R. J.; Higgs, D. R.; Cristea, I. M.; Urnov, F. D.; Zheng, D.; Allis, C. D., Distinct factors control histone variant H3.3 localization at specific genomic regions. *Cell* **2010,** *140* (5), 678-91.

Gorbsky, G. J., P. J. Sammak, and G. G. Borisy (1987). Chromosomes move poleward in anaphase along stationary microtubules that coordinately disassemble from their kinetochore ends. *J. Cell Biol.*, **104**:9–18.

Gorbsky, G. J., P. J. Sammak, and G. G. Borisy (1988). Microtubule dynamics and chromosome motion visualized in living anaphase cells. *J. Cell Biol.*, **106**:1185–1192.

Goutte-Gattat, D.; Shuaib, M.; Ouararhni, K.; Gautier, T.; Skoufias, D. A.; Hamiche, A.; Dimitrov, S., Phosphorylation of the CENP-A amino-terminus in mitotic centromeric chromatin is required for kinetochore function. *Proc Natl Acad Sci U S A* **2013,** *110* (21), 8579-84.

Greaves, I. K., D. Rangasamy, P. Ridgway, and D. J. Tremethick (2007). H2A.Z contributes to the unique 3D structure of the centromere. *Proc. Natl. Acad. Sci. USA*,

104:525–530.

Grewal, S. I.; Jia, S., Heterochromatin revisited. *Nat Rev Genet* **2007,** *8* (1), 35-46.
Grunstein, M. (1997). Histone acetylation in chromatin structure and transcription. *Nature*, **389**:349–352.

Guelen, L.; Pagie, L.; Brasset, E.; Meuleman, W.; Faza, M. B.; Talhout, W.; Eussen, B. H.; de
Haarhuis, J. H.; Elbatsh, A. M.; Rowland, B. D., Cohesin and its regulation: on the logic of X-shaped chromosomes. *Dev Cell* **2014,** *31* (1), 7-18.

Haering, C. H. and K. Nasmyth (2003). Building and breaking bridges between sister chromatids. *Bioessays*, **25**:1178–1191.

Hake, S. B. and C. D. Allis (2006). Histone H3 variants and their potential role in indexing mammalian genomes: the "H3 barcode hypothesis". *Proc. Natl. Acad. Sci. USA*, **103**:6428–6435.

Hake, S. B.; Garcia, B. A.; Kauer, M.; Baker, S. P.; Shabanowitz, J.; Hunt, D. F.; Allis, C. D., Serine 31 phosphorylation of histone variant H3.3 is specific to regions bordering centromeres in metaphase chromosomes. *Proc Natl Acad Sci U S A* **2005,** *102* (18), 6344-9.

Han, J. S.; Holland, A. J.; Fachinetti, D.; Kulukian, A.; Cetin, B.; Cleveland, D. W., Catalytic assembly of the mitotic checkpoint inhibitor BubR1-Cdc20 by a Mad2-induced functional switch in Cdc20. *Mol Cell* **2013,** *51* (1), 92-104.

Hannak, E., M. Kirkham, A. A. Hyman, and K. Oegema (2001). Aurora-A kinase is required for centrosome maturation in Caenorhabditis elegans. *J. Cell Biol.*, **155**:1109–1116.

Hans, F. and S. Dimitrov (2001). Histone H3 phosphorylation and cell division. *Oncogene*, **20**:3021–3027.

Hansen, J. C. (2002). Conformational dynamics of the chromatin fiber in solution: determinants, mechanisms, and functions. *Annu. Rev. Biophys. Biomol. Struct.*, **31**:361–392.

Hartman, P. G.; Chapman, G. E.; Moss, T.; Bradbury, E. M., Studies on the role and mode of operation of the very-lysine-rich histone H1 in eukaryote chromatin. The three structural regions of the histone H1 molecule. *Eur J Biochem* **1977,** *77* (1), 45-51.

Heald, R.; McKeon, F., Mutations of phosphorylation sites in lamin A that prevent nuclear lamina disassembly in mitosis. *Cell* **1990,** *61* (4), 579-89.

Hendriks, I. A.; D'Souza, R. C.; Yang, B.; Verlaan-de Vries, M.; Mann, M.; Vertegaal, A. C., Uncovering global SUMOylation signaling networks in a site-specific manner. *Nat Struct Mol Biol* **2014,** *21* (10), 927-36.

Henikoff, S. and A. Shilatifard (2011). Histone modification: cause or cog? *Trends Genet*., **27**:389–396.

Henikoff, S.; Henikoff, J. G., "Point" centromeres of Saccharomyces harbor single centromere-specific nucleosomes. *Genetics* **2012,** *190* (4), 1575-7.

Hermeking, H. and A. Benzinger (2006). 14-3-3 proteins in cell cycle regulation. *Semin. Cancer Biol*., **16**:183–192.

Hershko, A.; Ciechanover, A., The ubiquitin system. *Annu Rev Biochem* **1998,** *67*, 425-79.

Hinchcliffe, E. H.; Miller, F. J.; Cham, M.; Khodjakov, A.; Sluder, G., Requirement of a centrosomal activity for cell cycle progression through G1 into S phase. *Science* **2001,** *291* (5508), 1547-50.

Hines, K. A.; Cryderman, D. E.; Flannery, K. M.; Yang, H.; Vitalini, M. W.; Hazelrigg, T.; Mizzen, C. A.; Wallrath, L. L., Domains of heterochromatin protein 1 required for Drosophila melanogaster heterochromatin spreading. *Genetics* **2009,** *182* (4), 967-77.

Holland, A. J.; Fachinetti, D.; Han, J. S.; Cleveland, D. W., Inducible, reversible system for the rapid and complete degradation of proteins in mammalian cells. *Proc Natl Acad Sci U S A* **2012,** *109* (49), E3350-7.

Horowitz-Scherer, R. A.; Woodcock, C. L., Organization of interphase chromatin. *Chromosoma* **2006,** *115* (1), 1-14.

Howell, B. J., B. Moree, E. M. Farrar, S. Stewart, G. Fang, and E. D. Salmon (2004). Spindle checkpoint protein dynamics at kinetochores in living cells. *Curr. Biol*., **14**:953–964.

Hsu, J. Y.; Sun, Z. W.; Li, X.; Reuben, M.; Tatchell, K.; Bishop, D. K.; Grushcow, J. M.; Brame, C. J.; Caldwell, J. A.; Hunt, D. F.; Lin, R.; Smith, M. M.; Allis, C. D., Mitotic phosphorylation of histone H3 is governed by Ipl1/aurora kinase and Glc7/PP1 phosphatase in budding yeast and nematodes. *Cell* **2000,** *102* (3), 279-91.

Iemura, K.; Tanaka, K., Chromokinesin Kid and kinetochore kinesin CENP-E differentially support chromosome congression without end-on attachment to microtubules. *Nat Commun* **2015,** *6*, 6447.

Ikura, T.; Sugiyama, M.; Sato, M.; Kurumizaka, H., Structural basis of a nucleosome containing histone H2A.B/H2A.Bbd that transiently associates with reorganized chromatin. *Sci Rep* **2013,** *3*, 3510.

Jabs, E. W., C. M. Tuck-Muller, G. J. Anhalt, W. Earnshaw, R. A. Wise, and F. Wigley (1993). Cytogenetic survey in systemic sclerosis: correlation of aneuploidy with the presence of anticentromere antibodies. *Cytogenet. Cell Genet*., **63**:169–175.

Jacobson, R. H., A. G. Ladurner, D. S. King, and R. Tjian (2000). Structure and function

of a human TAFII250 double bromodomain module. *Science*, **288**:1422–1425.

Jansen, L. E. T., B. E. Black, D. R. Foltz, and D. W. Cleveland (2007). Propagation of centromeric chromatin requires exit from mitosis. *J. Cell Biol.*, **176**:795–805.

Joglekar, A. P., D. Bouck, K. Finley, X. Liu, Y. Wan, J. Berman, X. He, E. D. Salmon, and K. S. Bloom (2008). Molecular architecture of the kinetochore- microtubule attachment site is conserved between point and regional centromeres. *J. Cell Biol.*, **181**:587–594.

Joglekar, A. P., D. C. Bouck, J. N. Molk, K. S. Bloom, and E. D. Salmon (2006). Molecular architecture of a kinetochore-microtubule attachment site. *Nat. Cell Biol.*, **8**:581–585.

Joti, Y.; Hikima, T.; Nishino, Y.; Kamada, F.; Hihara, S.; Takata, H.; Ishikawa, T.; Maeshima, K., Chromosomes without a 30-nm chromatin fiber. *Nucleus* **2012,** *3* (5), 404-10.

Kadauke, S.; Blobel, G. A., Mitotic bookmarking by transcription factors. *Epigenetics Chromatin* **2013,** *6* (1), 6.

Kamakaka, R. T.; Biggins, S., Histone variants: deviants? *Genes Dev* **2005,** *19* (3), 295-310.

Kapoor, T. M., M. A. Lampson, P. Hergert, L. Cameron, D. Cimini, E. D. Salmon, B. F. McEwen, and A. Khodjakov (2006). Chromosomes can congress to the metaphase plate before biorientation. *Science*, **311**:388–391.

Karg, T.; Warecki, B.; Sullivan, W., Aurora B-mediated localized delays in nuclear envelope formation facilitate inclusion of late-segregating chromosome fragments. *Mol Biol Cell* **2015,** *26* (12), 2227-41.

Karpen, G. H. and R. C. Allshire (1997). The case for epigenetic effects on centromere identity and function. *Trends Genet.*, **13**:489–496.

Katan-Khaykovich, Y. and K. Struhl (2002). Dynamics of global histone acetylation and deacetylation in vivo: rapid restoration of normal histone acetylation status upon removal of activators and repressors. *Genes Dev.*, **16**:743–752.

Kato, H.; Jiang, J.; Zhou, B. R.; Rozendaal, M.; Feng, H.; Ghirlando, R.; Xiao, T. S.; Straight, A. F.; Bai, Y., A conserved mechanism for centromeric nucleosome recognition by centromere protein CENP-C. *Science* **2013,** *340* (6136), 1110-3.

Kelly, A. E. and H. Funabiki (2009). Correcting aberrant kinetochore microtubule attachments: an Aurora B-centric view. *Curr. Opin. Cell Biol.*, **21**:51–58.

Khochbin, S., Histone H1 diversity: bridging regulatory signals to linker histone function. *Gene* **2001,** *271* (1), 1-12.

Khodjakov, A. and C. L. Rieder (1996). Kinetochores moving away from their associated

pole do not exert a significant pushing force on the chromosome. *J. Cell Biol.*, **135**:315–327.

Khodjakov, A. and C. L. Rieder (1999). The sudden recruitment of gamma-tubulin to the centrosome at the onset of mitosis and its dynamic exchange throughout the cell cycle, do not require microtubules. *J. Cell Biol.*, **146**:585–596.

Khodjakov, A. and T. Kapoor (2005). Microtubule flux: what is it good for? *Curr. Biol.*, **15**:R966–R968.

Kim, H. S.; Vanoosthuyse, V.; Fillingham, J.; Roguev, A.; Watt, S.; Kislinger, T.; Treyer, A.; Carpenter, L. R.; Bennett, C. S.; Emili, A.; Greenblatt, J. F.; Hardwick, K. G.; Krogan, N. J.; Bähler, J.; Keogh, M. C., An acetylated form of histone H2A.Z regulates chromosome architecture in Schizosaccharomyces pombe. *Nat Struct Mol Biol* **2009,** *16* (12), 1286-93.

Kim, U. J.; Han, M.; Kayne, P.; Grunstein, M., Effects of histone H4 depletion on the cell cycle and transcription of Saccharomyces cerevisiae. *EMBO J* **1988,** *7* (7), 2211-9.

Kimura, K.; Hirano, M.; Kobayashi, R.; Hirano, T., Phosphorylation and activation of 13S condensin by Cdc2 in vitro. *Science* **1998,** *282* (5388), 487-90.

Kireeva, N., M. Lakonishok, I. Kireev, T. Hirano, and A. S. Belmont (2004). Visualization of early chromosome condensation: a hierarchical folding, axial glue model of chromosome structure. *J. Cell Biol.*, **166**:775–785.

Klare, K.; Weir, J. R.; Basilico, F.; Zimniak, T.; Massimiliano, L.; Ludwigs, N.; Herzog, F.; Musacchio, A., CENP-C is a blueprint for constitutive centromere-associated network assembly within human kinetochores. *J Cell Biol* **2015,** *210* (1), 11-22.

Klein, A.; Wessels, L.; de Laat, W.; van Steensel, B., Domain organization of human chromosomes revealed by mapping of nuclear lamina interactions. *Nature* **2008,** *453* (7197), 948-51.

Knowlton, A. L., W. Lan, and P. T. Stukenberg (2006). Aurora B is enriched at merotelic attachment sites, where it regulates MCAK. *Curr. Biol.*, **16**:1705–1710.

Kouzarides, T., Chromatin modifications and their function. *Cell* **2007,** *128* (4), 693-705.

Kunitoku, N., T. Sasayama, T. Marumoto, D. Zhang, S. Honda, O. Kobayashi, K. Hatakeyama, Y. Ushio, H. Saya, and T. Hirota (2003). CENP-A phosphorylation by Aurora-A in prophase is required for enrichment of Aurora-B at inner centromeres and for kinetochore function. *Dev. Cell*, **5**:853–864.

Kwok, B. H. and T. M. Kapoor (2007). Microtubule flux: drivers wanted. *Curr. Opin. Cell Biol.*, **19**:36–42.

Kwon, M. S.; Hori, T.; Okada, M.; Fukagawa, T., CENP-C is involved in chromosome segregation, mitotic checkpoint function, and kinetochore assembly. *Mol Biol Cell* **2007,** *18* (6), 2155-68.

Lachner, M. and T. Jenuwein (2002). The many faces of histone lysine methylation. *Curr. Opin. Cell Biol.*, **14**:286–298.

Lachner, M., R. J. O'Sullivan, and T. Jenuwein (2003). An epigenetic road map for histone lysine methylation. *J. Cell. Sci.*, **116**:2117–2124.

Lagana, A., J. F. Dorn, V. De Rop, A.-M. Ladouceur, A. S. Maddox, and P. S. Maddox (2010). A small GTPase molecular switch regulates epigenetic centromere maintenance by stabilizing newly incorporated CENP-A. *Nat. Cell Biol.*, **12**:1186– 1193.

Lampson, M. A., K. Renduchitala, A. Khodjakov, and T. M. Kapoor (2004). Correct- ing improper chromosome-spindle attachments during cell division. *Nat. Cell Biol.*, **6**:232–237.

Lampson, M. A.; Cheeseman, I. M., Sensing centromere tension: Aurora B and the regulation of kinetochore function. *Trends Cell Biol* **2011,** *21* (3), 133-40.

Lawrence, M.; Daujat, S.; Schneider, R., Lateral Thinking: How Histone Modifications Regulate Gene Expression. *Trends Genet* **2016,** *32* (1), 42-56.

Lee, S.-H., P. Itkin-Ansari, and F. Levine (2010). CENP-A, a protein required for chromosome segregation in mitosis, declines with age in islet but not exocrine cells. *Aging (Albany NY)*, **2**:785–790.

Lindon, C.; Grant, R.; Min, M., Ubiquitin-Mediated Degradation of Aurora Kinases. *Front Oncol* **2015,** *5*, 307.

Lioutas, A.; Vernos, I., Aurora A kinase and its substrate TACC3 are required for central spindle assembly. *EMBO Rep* **2013,** *14* (9), 829-36.

Lipp, J. J., T. Hirota, I. Poser, and J.-M. Peters (2007). Aurora B controls the association of condensin I but not condensin II with mitotic chromosomes. *J. Cell. Sci.*, **120**:1245–1255.

Liu, D. and M. A. Lampson (2009). Regulation of kinetochore-microtubule attachments by Aurora B kinase. *Biochem. Soc. Trans.*, **37**:976–980.

Logsdon, G. A.; Barrey, E. J.; Bassett, E. A.; DeNizio, J. E.; Guo, L. Y.; Panchenko, T.; Dawicki-McKenna, J. M.; Heun, P.; Black, B. E., Both tails and the centromere targeting domain of CENP-A are required for centromere establishment. *J Cell Biol* **2015,** *208* (5), 521-31.

Luger, K., A. W. Mäder, R. K. Richmond, D. F. Sargent, and T. J. Richmond (1997). Crystal structure of the nucleosome core particle at 2.8 A resolution. *Nature*, **389**:251–260.

Macůrek, L., A. Lindqvist, D. Lim, M. A. Lampson, R. Klompmaker, R. Freire, C. Clouin,

S. S. Taylor, M. B. Yaffe, and R. H. Medema (2008). Polo-like kinase-1 is activated by aurora A to promote checkpoint recovery. *Nature*, **455**:119–123.

Macůrek, L.; Lindqvist, A.; Voets, O.; Kool, J.; Vos, H. R.; Medema, R. H., Wip1 phosphatase is associated with chromatin and dephosphorylates gammaH2AX to promote checkpoint inhibition. *Oncogene* **2010,** *29* (15), 2281-91.

Maehara, K., K. Takahashi, and S. Saitoh (2010). CENP-A reduction induces a p53-dependent cellular senescence response to protect cells from executing defective mi- toses. *Mol. Cell. Biol.*, **30**:2090–2104.

Maeshima, K.; Hihara, S.; Takata, H., New insight into the mitotic chromosome structure: irregular folding of nucleosome fibers without 30-nm chromatin structure. *Cold Spring Harb Symp Quant Biol* **2010,** *75*, 439-44.

Maeshima, K.; Laemmli, U. K., A two-step scaffolding model for mitotic chromosome assembly. *Dev Cell* **2003,** *4* (4), 467-80.

Maeshima, K.; Rogge, R.; Tamura, S.; Joti, Y.; Hikima, T.; Szerlong, H.; Krause, C.; Herman, J.; Seidel, E.; DeLuca, J.; Ishikawa, T.; Hansen, J. C., Nucleosomal arrays self-assemble into supramolecular globular structures lacking 30-nm fibers. *EMBO J* **2016,** *35* (10), 1115-32.

Mahoney, N. M., G. Goshima, A. D. Douglass, and R. D. Vale (2006). Making microtubules and mitotic spindles in cells without functional centrosomes. *Curr. Biol.*, **16**:564–569.

Maiato, H.; Afonso, O.; Matos, I., A chromosome separation checkpoint: A midzone Aurora B gradient mediates a chromosome separation checkpoint that regulates the anaphase-telophase transition. *Bioessays* **2015,** *37* (3), 257-66.

Malik, H. S.; Henikoff, S., Phylogenomics of the nucleosome. *Nat Struct Biol* **2003,** *10* (11), 882-91.

Malumbres, M.; Harlow, E.; Hunt, T.; Hunter, T.; Lahti, J. M.; Manning, G.; Morgan, D. O.; Tsai, L. H.; Wolgemuth, D. J., Cyclin-dependent kinases: a family portrait. *Nat Cell Biol* **2009,** *11* (11), 1275-6.

Maresca, T. J.; Salmon, E. D., Welcome to a new kind of tension: translating kinetochore mechanics into a wait-anaphase signal. *J Cell Sci* **2010,** *123* (Pt 6), 825-35.

Marfella, C. G. A. and A. N. Imbalzano (2007). The Chd family of chromatin remodelers. *Mutat. Res.*, **618**:30–40.

Margolis, R. L. and L. Wilson (1998). Microtubule treadmilling: what goes around comes around. *Bioessays*, **20**:830–836.

Marko, J. F. (2008). Micromechanical studies of mitotic chromosomes. *Chromosome Res.*,

16:469–497.

Marshall, O. J., A. C. Chueh, L. H. Wong, and K. H. A. Choo (2008). Neocentromeres: new insights into centromere structure, disease development, and karyotype evolution. *Am. J. Hum. Genet.*, **82**:261–282.

Marzluff, W. F., P. Gongidi, K. R. Woods, J. Jin, and L. J. Maltais (2002). The human and mouse replication-dependent histone genes. *Genomics*, **80**:487–498.

Mathew, V.; Pauleau, A. L.; Steffen, N.; Bergner, A.; Becker, P. B.; Erhardt, S., The histone-fold protein CHRAC14 influences chromatin composition in response to DNA damage. *Cell Rep* **2014,** *7* (2), 321-30.

McAinsh, A. D., J. D. Tytell, and P. K. Sorger (2003). Structure, function, and regulation of budding yeast kinetochores. *Annu. Rev. Cell Dev. Biol.*, **19**:519–539.

McEwen, B. F., Y. Dong, and K. J. VandenBeldt (2007). Using electron microscopy to understand functional mechanisms of chromosome alignment on the mitotic spindle. *Methods Cell Biol.*, **79**:259–293.

McGovern, S. L.; Qi, Y.; Pusztai, L.; Symmans, W. F.; Buchholz, T. A., Centromere protein-A, an essential centromere protein, is a prognostic marker for relapse in estrogen receptor-positive breast cancer. *Breast Cancer Res* **2012,** *14* (3), R72.

McIntosh, J. R., E. L. Grishchuk, and R. R. West (2002). Chromosome-microtubule interactions during mitosis. *Annu. Rev. Cell Dev. Biol.*, **18**:193–219.

McKim, K. S. and R. S. Hawley (1995). Chromosomal control of meiotic cell division. *Science*, **270**:1595–1601.

McKinley, K. L.; Cheeseman, I. M., Polo-like kinase 1 licenses CENP-A deposition at centromeres. *Cell* **2014,** *158* (2), 397-411.

McKinley, K. L.; Sekulic, N.; Guo, L. Y.; Tsinman, T.; Black, B. E.; Cheeseman, I. M., The CENP-L-N Complex Forms a Critical Node in an Integrated Meshwork of Interactions at the Centromere-Kinetochore Interface. *Mol Cell* **2015,** *60* (6), 886-98.

McKittrick, E., P. R. Gafken, K. Ahmad, and S. Henikoff (2004). Histone H3.3 is enriched in covalent modifications associated with active chromatin. *Proc. Natl. Acad. Sci. USA*, **101**:1525–1530.

Meistrich, M. L., L. R. Bucci, P. K. Trostle-Weige, and W. A. Brock (1985). Histone variants in rat spermatogonia and primary spermatocytes. *Dev. Biol.*, **112**:230–240.

Mellone, B. G., W. Zhang, and G. H. Karpen (2009). Frodos found: Behold the CENP-a "Ring" bearers. *Cell*, **137**:409–412.

Melters, D. P.; Nye, J.; Zhao, H.; Dalal, Y., Chromatin Dynamics in Vivo: A Game of Musical Chairs. *Genes (Basel)* **2015,** *6* (3), 751-76.

Meneghini, M. D., M. Wu, and H. D. Madhani (2003). Conserved histone variant H2A.Z protects euchromatin from the ectopic spread of silent heterochromatin. *Cell*, **112**:725–736

Mietton, F., A. K. Sengupta, A. Molla, G. Picchi, S. Barral, L. Heliot, T. Grange, A. Wutz, and S. Dimitrov (2009). Weak but uniform enrichment of the histone variant macroH2A1 along the inactive X chromosome. *Mol. Cell. Biol.*, **29**:150–156.

Millar, C. B.; Grunstein, M., Genome-wide patterns of histone modifications in yeast. *Nat Rev Mol Cell Biol* **2006,** *7* (9), 657-66.

Minoshima, Y., T. Kawashima, K. Hirose, Y. Tonozuka, A. Kawajiri, Y. C. Bao, X. Deng, M. Tatsuka, S. Narumiya, and W. S. May (2003). Phosphorylation by aurora B converts MgcRacGAP to a RhoGAP during cytokinesis. *Dev. Cell*, **4**:549–560.

Misteli, T.; Gunjan, A.; Hock, R.; Bustin, M.; Brown, D. T., Dynamic binding of histone H1 to chromatin in living cells. *Nature* **2000,** *408* (6814), 877-81.

Mizuguchi, G., H. Xiao, J. Wisniewski, M. M. Smith, and C. Wu (2007). Nonhistone Scm3 and histones CenH3-H4 assemble the core of centromere-specific nucleosomes. *Cell*, **129**:1153–1164.

Mohrmann, L. and C. P. Verrijzer (2005). Composition and functional specificity of SWI2/SNF2 class chromatin remodeling complexes. *Biochim. Biophys. Acta*, **1681**:59–73.

Montellier, E.; Boussouar, F.; Rousseaux, S.; Zhang, K.; Buchou, T.; Fenaille, F.; Shiota, H.; Debernardi, A.; Héry, P.; Curtet, S.; Jamshidikia, M.; Barral, S.; Holota, H.; Bergon, A.; Lopez, F.; Guardiola, P.; Pernet, K.; Imbert, J.; Petosa, C.; Tan, M.; Zhao, Y.; Gérard, M.; Khochbin, S., Chromatin-to-nucleoprotamine transition is controlled by the histone H2B variant TH2B. *Genes Dev* **2013,** *27* (15), 1680-92.

Morey, L., K. Barnes, Y. Chen, M. Fitzgerald-Hayes, and R. E. Baker (2004). The histone fold domain of Cse4 is sufficient for CEN targeting and propagation of active centromeres in budding yeast. *Eukaryotic Cell*, **3**:1533–1543.

Müller, S.; Montes de Oca, R.; Lacoste, N.; Dingli, F.; Loew, D.; Almouzni, G., Phosphorylation and DNA binding of HJURP determine its centromeric recruitment and function in CenH3(CENP-A) loading. *Cell Rep* **2014,** *8* (1), 190-203.

Murray, A. W., Recycling the cell cycle: cyclins revisited. *Cell* **2004,** *116* (2), 221-34.

Musacchio, A. and E. D. Salmon (2007). The spindle-assembly checkpoint in space and time. *Nat. Rev. Mol. Cell Biol.*, **8**:379–393.

Mutskov, V., D. Gerber, D. Angelov, J. Ausio, J. Workman, and S. Dimitrov (1998). Persistent interactions of core histone tails with nucleosomal DNA following acetyla- tion and transcription factor binding. *Mol. Cell. Biol.*, **18**:6293–6304.

Nagpal, H.; Hori, T.; Furukawa, A.; Sugase, K.; Osakabe, A.; Kurumizaka, H.; Fukagawa, T., Dynamic changes in CCAN organization through CENP-C during cell-cycle progression. *Mol Biol Cell* **2015,** *26* (21), 3768-76.

Naumova, N.; Imakaev, M.; Fudenberg, G.; Zhan, Y.; Lajoie, B. R.; Mirny, L. A.; Dekker, J., Organization of the mitotic chromosome. *Science* **2013,** *342* (6161), 948-53.

Nekrasov, M.; Amrichova, J.; Parker, B. J.; Soboleva, T. A.; Jack, C.; Williams, R.; Huttley, G. A.; Tremethick, D. J., Histone H2A.Z inheritance during the cell cycle and its impact on promoter organization and dynamics. *Nat Struct Mol Biol* **2012,** *19* (11), 1076-83.

Neumann, P., H. Yan, and J. Jiang (2007). The centromeric retrotransposons of rice are transcribed and differentially processed by RNA interference. *Genetics*, **176**:749–761.

Neurohr, G.; Naegeli, A.; Titos, I.; Theler, D.; Greber, B.; Díez, J.; Gabaldón, T.; Mendoza, M.; Barral, Y., A midzone-based ruler adjusts chromosome compaction to anaphase spindle length. *Science* **2011,** *332* (6028), 465-8.

Nijman, S. M.; Luna-Vargas, M. P.; Velds, A.; Brummelkamp, T. R.; Dirac, A. M.; Sixma, T. K.; Bernards, R., A genomic and functional inventory of deubiquitinating enzymes. *Cell* **2005,** *123* (5), 773-86.

Nilsson, I.; Hoffmann, I., Cell cycle regulation by the Cdc25 phosphatase family. *Prog Cell Cycle Res* **2000,** *4*, 107-14.

Nishimura, K.; Fukagawa, T.; Takisawa, H.; Kakimoto, T.; Kanemaki, M., An auxin-based degron system for the rapid depletion of proteins in nonplant cells. *Nat Methods* **2009,** *6* (12), 917-22.

Nishino, T.; Rago, F.; Hori, T.; Tomii, K.; Cheeseman, I. M.; Fukagawa, T., CENP-T provides a structural platform for outer kinetochore assembly. *EMBO J* **2013,** *32* (3), 424-36.

Nishino, Y.; Eltsov, M.; Joti, Y.; Ito, K.; Takata, H.; Takahashi, Y.; Hihara, S.; Frangakis, A. S.; Imamoto, N.; Ishikawa, T.; Maeshima, K., Human mitotic chromosomes consist predominantly of irregularly folded nucleosome fibres without a 30-nm chromatin structure. *EMBO J* **2012,** *31* (7), 1644-53.

Nogales, E. (2001). Structural insight into microtubule function. *Annu Rev Biophys Biomol Struct*, **30**:397–420.

Nora, E. P.; Lajoie, B. R.; Schulz, E. G.; Giorgetti, L.; Okamoto, I.; Servant, N.; Piolot, T.; van Berkum, N. L.; Meisig, J.; Sedat, J.; Gribnau, J.; Barillot, E.; Blüthgen, N.; Dekker, J.; Heard, E., Spatial partitioning of the regulatory landscape of the X-inactivation centre. *Nature* **2012,** *485* (7398), 381-5.

O'Connell, M. J., M. J. E. Krien, and T. Hunter (2003). Never say never. The NIMA-

related protein kinases in mitotic control. *Trends Cell Biol*., **13**:221–228.

Ogiyama, Y.; Ohno, Y.; Kubota, Y.; Ishii, K., Epigenetically induced paucity of histone H2A.Z stabilizes fission-yeast ectopic centromeres. *Nat Struct Mol Biol* **2013,** *20* (12), 1397-406.

Ohkuni, K.; Kitagawa, K., Endogenous transcription at the centromere facilitates centromere activity in budding yeast. *Curr Biol* **2011,** *21* (20), 1695-703.

Olins, D. E. and A. L. Olins (2003). Chromatin history: our view from the bridge. *Nat. Rev. Mol. Cell Biol*., **4**:809–814.

Osley, M. A. (1991). The regulation of histone synthesis in the cell cycle. *Annu. Rev. Biochem*., **60**:827–861.

Palmer, D. K., K. O'Day, M. H. Wener, B. S. Andrews, and R. L. Margolis (1987). A 17-kD centromere protein (CENP-A) copurifies with nucleosome core particles and with histones. *J. Cell Biol*., **104**:805–815.

Parker, L. L.; Piwnica-Worms, H., Inactivation of the p34cdc2-cyclin B complex by the human WEE1 tyrosine kinase. *Science* **1992,** *257* (5078), 1955-7.

Pavri, R., B. Zhu, G. Li, P. Trojer, S. Mandal, A. Shilatifard, and D. Reinberg (2006). Histone H2B monoubiquitination functions cooperatively with FACT to regulate elongation by RNA polymerase II. *Cell*, **125**:703–717.

Pehrson, J. R.; Changolkar, L. N.; Costanzi, C.; Leu, N. A., Mice without macroH2A histone variants. *Mol Cell Biol* **2014,** *34* (24), 4523-33.

Perpelescu, M. and T. Fukagawa (2011). The ABCs of CENPs. *Chromosoma*, **120**:425–446.

Perpelescu, M., N. Nozaki, C. Obuse, H. Yang, and K. Yoda (2009). Active establishment of centromeric CENP-A chromatin by RSF complex. *J. Cell Biol*., **185**:397– 407.

Pesenti, M. E.; Weir, J. R.; Musacchio, A., Progress in the structural and functional characterization of kinetochores. *Curr Opin Struct Biol* **2016,** *37*, 152-63.

Petronczki, M., P. Lénárt, and J.-M. Peters (2008). Polo on the Rise-from Mitotic Entry to Cytokinesis with Plk1. *Dev. Cell*, **14**:646–659.

Pfarr, C. M., M. Coue, P. M. Grissom, T. S. Hays, M. E. Porter, and J. R. McIntosh (1990). Cytoplasmic dynein is localized to kinetochores during mitosis. *Nature*, **345**:263–265.

Pickersgill, H.; Kalverda, B.; de Wit, E.; Talhout, W.; Fornerod, M.; van Steensel, B., Characterization of the Drosophila melanogaster genome at the nuclear lamina. *Nat Genet* **2006,** *38* (9), 1005-14.

Piehl, M., U. S. Tulu, P. Wadsworth, and L. Cassimeris (2004). Centrosome maturation:

measurement of microtubule nucleation throughout the cell cycle by using GFP-tagged EB1. *Proc. Natl. Acad. Sci. U.S.A.*, **101**:1584–1588.

Pluta, A. F.; Mackay, A. M.; Ainsztein, A. M.; Goldberg, I. G.; Earnshaw, W. C., The centromere: hub of chromosomal activities. *Science* **1995,** *270* (5242), 1591-4.

Poirier, M. G.; Marko, J. F., Micromechanical studies of mitotic chromosomes. *J Muscle Res Cell Motil* **2002,** *23* (5-6), 409-31.

Poirier, M. G.; Marko, J. F., Mitotic chromosomes are chromatin networks without a mechanically contiguous protein scaffold. *Proc Natl Acad Sci U S A* **2002,** *99* (24), 15393-7.

Przewloka, M. R.; Venkei, Z.; Bolanos-Garcia, V. M.; Debski, J.; Dadlez, M.; Glover, D. M., CENP-C is a structural platform for kinetochore assembly. *Curr Biol* **2011,** *21* (5), 399-405.

Raisner, R. M.; Hartley, P. D.; Meneghini, M. D.; Bao, M. Z.; Liu, C. L.; Schreiber, S. L.; Rando, O. J.; Madhani, H. D., Histone variant H2A.Z marks the 5' ends of both active and inactive genes in euchromatin. *Cell* **2005,** *123* (2), 233-48.

Rangasamy, D.; Greaves, I.; Tremethick, D. J., RNA interference demonstrates a novel role for H2A.Z in chromosome segregation. *Nat Struct Mol Biol* **2004,** *11* (7), 650-5.

Rao, S. S.; Huntley, M. H.; Durand, N. C.; Stamenova, E. K.; Bochkov, I. D.; Robinson, J. T.; Sanborn, A. L.; Machol, I.; Omer, A. D.; Lander, E. S.; Aiden, E. L., A 3D map of the human genome at kilobase resolution reveals principles of chromatin looping. *Cell* **2014,** *159* (7), 1665-80.

Reboutier, D.; Troadec, M. B.; Cremet, J. Y.; Chauvin, L.; Guen, V.; Salaun, P.; Prigent, C., Aurora A is involved in central spindle assembly through phosphorylation of Ser 19 in P150Glued. *J Cell Biol* **2013,** *201* (1), 65-79.

Rieder, C. L., A. Schultz, R. Cole, and G. Sluder (1994). Anaphase onset in vertebrate somatic cells is controlled by a checkpoint that monitors sister kinetochore attachment to the spindle. *J. Cell Biol.*, **127**:1301–1310.

Rogakou, E. P., D. R. Pilch, A. H. Orr, V. S. Ivanova, and W. M. Bonner (1998). DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139. *J. Biol. Chem.*, **273**:5858–5868.

Roque, A., I. Iloro, I. Ponte, J. L. R. Arrondo, and P. Suau (2005). DNA-induced secondary structure of the carboxyl-terminal domain of histone H1. *J. Biol. Chem.*, **280**:32141–32147.

Rosenblatt, J. (2005). Spindle assembly: asters part their separate ways. *Nat. Cell Biol.*, **7**:219–222.

Roshak, A. K., E. A. Capper, C. Imburgia, J. Fornwald, G. Scott, and L. A. Marshall

(2000). The human polo-like kinase, PLK, regulates cdc2/cyclin B through phosphorylation and activation of the cdc25C phosphatase. *Cell. Signal.*, **12**:405–411.

Rošić, S.; Erhardt, S., No longer a nuisance: long non-coding RNAs join CENP-A in epigenetic centromere regulation. *Cell Mol Life Sci* **2016,** *73* (7), 1387-98.

Rošić, S.; Köhler, F.; Erhardt, S., Repetitive centromeric satellite RNA is essential for kinetochore formation and cell division. *J Cell Biol* **2014,** *207* (3), 335-49.

Roulland, Y.; Ouararhni, K.; Naidenov, M.; Ramos, L.; Shuaib, M.; Syed, S. H.; Lone, I. N.; Boopathi, R.; Fontaine, E.; Papai, G.; Tachiwana, H.; Gautier, T.; Skoufias, D.; Padmanabhan, K.; Bednar, J.; Kurumizaka, H.; Schultz, P.; Angelov, D.; Hamiche, A.; Dimitrov, S., The Flexible Ends of CENP-A Nucleosome Are Required for Mitotic Fidelity. *Mol Cell* **2016,** *63* (4), 674-85.

Saffery, R., H. Sumer, S. Hassan, L. H. Wong, J. M. Craig, K. Todokoro, M. Anderson, A. Stafford, and K. H. A. Choo (2003). Transcription within a functional human centromere. *Mol. Cell*, **12**:509–516.

Sandman, K.; Krzycki, J. A.; Dobrinski, B.; Lurz, R.; Reeve, J. N., HMf, a DNA-binding protein isolated from the hyperthermophilic archaeon Methanothermus fervidus, is most closely related to histones. *Proc Natl Acad Sci U S A* **1990,** *87* (15), 5788-91.

Santos-Rosa, H.; Schneider, R.; Bannister, A. J.; Sherriff, J.; Bernstein, B. E.; Emre, N. C.; Schreiber, S. L.; Mellor, J.; Kouzarides, T., Active genes are tri-methylated at K4 of histone H3. *Nature* **2002,** *419* (6905), 407-11.

Sarcinella, E.; Zuzarte, P. C.; Lau, P. N.; Draker, R.; Cheung, P., Monoubiquitylation of H2A.Z distinguishes its association with euchromatin or facultative heterochromatin. *Mol Cell Biol* **2007,** *27* (18), 6457-68.

Sato, H.; Masuda, F.; Takayama, Y.; Takahashi, K.; Saitoh, S., Epigenetic inactivation and subsequent heterochromatinization of a centromere stabilize dicentric chromosomes. *Curr Biol* **2012,** *22* (8), 658-67.

Schindler, K.; Davydenko, O.; Fram, B.; Lampson, M. A.; Schultz, R. M., Maternally recruited Aurora C kinase is more stable than Aurora B to support mouse oocyte maturation and early development. *Proc Natl Acad Sci U S A* **2012,** *109* (33), E2215-22.

Schumacher, J. M.; Golden, A.; Donovan, P. J., AIR-2: An Aurora/Ipl1-related protein kinase associated with chromosomes and midbody microtubules is required for polar body extrusion and cytokinesis in Caenorhabditis elegans embryos. *J Cell Biol* **1998,** *143* (6), 1635-46.

Screpanti, E., A. De Antoni, G. M. Alushin, A. Petrovic, T. Melis, E. Nogales, and A. Musacchio (2011). Direct binding of cenp-C to the mis12 complex joins the inner and outer kinetochore. *Curr. Biol.*, **21**:391–398.

Seki, A., J. A. Coppinger, C.-Y. Jang, J. R. Yates, and G. Fang (2008). Bora and the kinase Aurora a cooperatively activate the kinase Plk1 and control mitotic entry. *Science*, **320**:1655–1658.

Sekulic, N., E. A. Bassett, D. J. Rogers, and B. E. Black (2010). The structure of (CENP-A-H4)(2) reveals physical features that mark centromeres. *Nature*, **467**:347– 351.

Sexton, T.; Yaffe, E.; Kenigsberg, E.; Bantignies, F.; Leblanc, B.; Hoichman, M.; Parrinello, H.; Tanay, A.; Cavalli, G., Three-dimensional folding and functional organization principles of the Drosophila genome. *Cell* **2012,** *148* (3), 458-72.

Shah, J. V., E. Botvinick, Z. Bonday, F. Furnari, M. Berns, and D. W. Cleveland (2004). Dynamics of centromere and kinetochore proteins; implications for checkpoint signaling and silencing. *Curr. Biol.*, **14**:942–952.

Shang, C., T. R. Hazbun, I. M. Cheeseman, J. Aranda, S. Fields, D. G. Drubin, and G. Barnes (2003). Kinetochore protein interactions and their regulation by the Aurora kinase Ipl1p. *Mol. Biol. Cell*, **14**:3342–3355.

Shaytan, A. K.; Armeev, G. A.; Goncearenco, A.; Zhurkin, V. B.; Landsman, D.; Panchenko, A. R., Coupling between Histone Conformations and DNA Geometry in Nucleosomes on a Microsecond Timescale: Atomistic Insights into Nucleosome Functions. *J Mol Biol* **2016,** *428* (1), 221-37.

Shaytan, A. K.; Landsman, D.; Panchenko, A. R., Nucleosome adaptability conferred by sequence and structural variations in histone H2A-H2B dimers. *Curr Opin Struct Biol* **2015,** *32*, 48-57.

Shelby, R. D., O. Vafa, and K. F. Sullivan (1997). Assembly of CENP-A into centromeric chromatin requires a cooperative array of nucleosomal DNA contact sites. *J. Cell Biol.*, **136**:501–513.

Shi, Y.; Lan, F.; Matson, C.; Mulligan, P.; Whetstine, J. R.; Cole, P. A.; Casero, R. A., Histone demethylation mediated by the nuclear amine oxidase homolog LSD1. *Cell* **2004,** *119* (7), 941-53.

Shuaib, M., K. Ouararhni, S. Dimitrov, and A. Hamiche (2010). HJURP binds CENP-A via a highly conserved N-terminal domain and mediates its deposition at centromeres. *Proc. Natl. Acad. Sci. U.S.A.*, **107**:1349–1354.

Silva, M. C.; Bodor, D. L.; Stellfox, M. E.; Martins, N. M.; Hochegger, H.; Foltz, D. R.; Jansen, L. E., Cdk activity couples epigenetic centromere inheritance to cell cycle progression. *Dev Cell* **2012,** *22* (1), 52-63.

Sotiriou, C.; Desmedt, C., Gene expression profiling in breast cancer. *Ann Oncol* **2006,** *17 Suppl 10*, x259-62.

Sterner, D. E. and S. L. Berger (2000). Acetylation of histones and transcription-related factors. *Microbiol. Mol. Biol. Rev.*, **64**:435–459.

Steuer, E. R., L. Wordeman, T. A. Schroer, and M. P. Sheetz (1990). Localization of cytoplasmic dynein to mitotic spindles and kinetochores. *Nature*, **345**:266–268.

Strahl, B. D. and C. D. Allis (2000). The language of covalent histone modifications. *Nature*, **403**:41–45.

Sugimoto, K.; Yata, H.; Muro, Y.; Himeno, M., Human centromere protein C (CENP-C) is a DNA-binding protein which possesses a novel DNA-binding motif. *J Biochem* **1994,** *116* (4), 877-81.

Sullivan, B. A. and G. H. Karpen (2004). Centromeric chromatin exhibits a histone modification pattern that is distinct from both euchromatin and heterochromatin. *Nat. Struct. Mol. Biol.*, **11**:1076–1083.

Sullivan, B. A., M. D. Blower, and G. H. Karpen (2001). Determining centromere identity: cyclical stories and forking paths. *Nat. Rev. Genet.*, **2**:584–596.

Sullivan, K. F., M. Hechenberger, and K. Masri (1994). Human CENP-A contains a histone H3 related histone fold domain that is required for targeting to the centromere. *J. Cell Biol.*, **127**:581–592.

Suto, R. K.; Clarkson, M. J.; Tremethick, D. J.; Luger, K., Crystal structure of a nucleosome core particle containing the variant histone H2A.Z. *Nat Struct Biol* **2000,** *7* (12), 1121-4.

Szenker, E., D. Ray-Gallet, and G. Almouzni (2011). The double face of the histone variant H3.3. *Cell Res.*, **21**:421–434.

Tachiwana, H., W. Kagawa, A. Osakabe, K. Kawaguchi, T. Shiga, Y. Hayashi- Takanaka, H. Kimura, and H. Kurumizaka (2010). Structural basis of instability of the nucleosome containing a testis-specific histone variant, human H3T. *Proc. Natl. Acad. Sci. U.S.A.*, **107**:10454–10459.

Tachiwana, H., W. Kagawa, T. Shiga, A. Osakabe, Y. Miya, K. Saito, Y. Hayashi-Takanaka, T. Oda, M. Sato, and S.-Y. Park (2011a). Crystal structure of the human centromeric nucleosome containing CENP-A. *Nature*, **476**:232–235.

Takayama, Y., H. Sato, S. Saitoh, Y. Ogiyama, F. Masuda, and K. Takahashi (2008). Biphasic incorporation of centromeric histone CENP-A in fission yeast. *Mol. Biol. Cell*, **19**:682–690.

Talbert, P. B.; Ahmad, K.; Almouzni, G.; Ausió, J.; Berger, F.; Bhalla, P. L.; Bonner, W. M.; Cande, W. Z.; Chadwick, B. P.; Chan, S. W.; Cross, G. A.; Cui, L.; Dimitrov, S. I.; Doenecke, D.; Eirin-López, J. M.; Gorovsky, M. A.; Hake, S. B.; Hamkalo, B. A.; Holec, S.; Jacobsen, S. E.; Kamieniarz, K.; Khochbin, S.; Ladurner, A. G.; Landsman, D.; Latham, J. A.; Loppin, B.; Malik, H. S.; Marzluff, W. F.; Pehrson, J. R.; Postberg, J.; Schneider, R.; Singh, M. B.; Smith, M. M.; Thompson, E.; Torres-Padilla, M. E.; Tremethick, D. J.; Turner, B. M.; Waterborg, J. H.; Wollmann, H.; Yelagandula, R.; Zhu, B.; Henikoff, S., A unified phylogeny-based nomenclature for histone variants. *Epigenetics Chromatin* **2012,** *5*, 7.

Tan, M.; Luo, H.; Lee, S.; Jin, F.; Yang, J. S.; Montellier, E.; Buchou, T.; Cheng, Z.; Rousseaux, S.; Rajagopal, N.; Lu, Z.; Ye, Z.; Zhu, Q.; Wysocka, J.; Ye, Y.; Khochbin, S.; Ren, B.; Zhao, Y., Identification of 67 histone marks and histone lysine crotonylation as a new type of histone modification. *Cell* **2011,** *146* (6), 1016-28.

Tanaka, T. U., N. Rachidi, C. Janke, G. Pereira, M. Galova, E. Schiebel, M. J. R. Stark, and K. Nasmyth (2002). Evidence that the Ipl1-Sli15 (Aurora kinase-INCENP) complex promotes chromosome bi-orientation by altering kinetochore-spindle pole con- nections. *Cell*, **108**:317–329.

Tang, C. J.; Lin, C. Y.; Tang, T. K., Dynamic localization and functional implications of Aurora-C kinase during male mouse meiosis. *Dev Biol* **2006,** *290* (2), 398-410.

Thiriet, C. and J. J. Hayes (2005). Chromatin in need of a fix: phosphorylation of H2AX connects chromatin to DNA repair. *Mol. Cell*, **18**:617–622.

Thoma, F.; Koller, T.; Klug, A., Involvement of histone H1 in the organization of the nucleosome and of the salt-dependent superstructures of chromatin. *J Cell Biol* **1979,** *83* (2 Pt 1), 403-27.

Tomonaga, T., K. Matsushita, S. Yamaguchi, T. Oohashi, H. Shimada, T. Ochiai, K. Yoda, and F. Nomura (2003). Overexpression and mistargeting of centromere protein-A in human primary colorectal cancer. *Cancer Res.*, **63**:3511–3516.

Torras-Llort, M., S. Medina-Giró, O. Moreno-Moreno, and F. Azorín (2010). A conserved arginine-rich motif within the hypervariable N-domain of Drosophila cen- tromeric histone H3 (CenH3) mediates BubR1 recruitment. *PLoS ONE*, **5**:e13747.

Urbani, L. and T. Stearns (1999). The centrosome. *Curr. Biol.*, **9**:R315–R317.Van Holde, K. E., J. R. Allen, K. Tatchell, W. O. Weischet, and D. Lohr (1980).

Van Hooser, A. A., I. I. Ouspenski, H. C. Gregson, D. A. Starr, T. J. Yen, M. L. Goldberg, K. Yokomori, W. C. Earnshaw, K. F. Sullivan, and B. R. Brinkley (2001). Specification of kinetochore-forming chromatin by the histone H3 variant CENP-A. *J. Cell Sci.*, **114**:3529–3542.

Van Steensel, B.; Braunschweig, U.; Filion, G. J.; Chen, M.; van Bemmel, J. G.; Ideker, T., Bayesian network analysis of targeting interactions in chromatin. *Genome Res* **2010,** *20* (2), 190-200.

Vardabasso, C.; Hasson, D.; Ratnakumar, K.; Chung, C. Y.; Duarte, L. F.; Bernstein, E., Histone variants: emerging players in cancer biology. *Cell Mol Life Sci* **2014,** *71* (3), 379-404.

Vermaak, D., H. S. Hayden, and S. Henikoff (2002). Centromere targeting element within the histone fold domain of Cid. *Mol. Cell. Biol.*, **22**:7553–7561.

Vitaliano-Prunier, A., A. Menant, M. Hobeika, V. Géli, C. Gwizdek, and C. Dargemont (2008). Ubiquitylation of the COMPASS component Swd2 links H2B ubiquitylation to H3K4 trimethylation. *Nat. Cell Biol.*, **10**:1365–1371.

Wang, H., L. Wang, H. Erdjument-Bromage, M. Vidal, P. Tempst, R. S. Jones, and Y. Zhang (2004). Role of histone H2A ubiquitination in Polycomb silencing. *Nature*, **431**:873–878.

Wang, Z.; Zang, C.; Rosenfeld, J. A.; Schones, D. E.; Barski, A.; Cuddapah, S.; Cui, K.; Roh, T. Y.; Peng, W.; Zhang, M. Q.; Zhao, K., Combinatorial patterns of histone acetylations and methylations in the human genome. *Nat Genet* **2008,** *40* (7), 897-903.

Weber, C. M.; Ramachandran, S.; Henikoff, S., Nucleosomes are context-specific, H2A.Z-modulated barriers to RNA polymerase. *Mol Cell* **2014,** *53* (5), 819-30.

Wei, R. R., J. Al-Bassam, and S. C. Harrison (2007). The Ndc80/HEC1 complex is a contact point for kinetochore-microtubule attachment. *Nat. Struct. Mol. Biol.*, **14**:54–59.

Wei, R. R., J. R. Schnell, N. A. Larsen, P. K. Sorger, J. J. Chou, and S. C. Harrison (2006). Structure of a central component of the yeast kinetochore: the Spc24p/Spc25p globular domain. *Structure*, **14**:1003–1009.

Welburn, J. P. I. and I. M. Cheeseman (2008). Toward a molecular structure of the eukaryotic kinetochore. *Dev. Cell*, **15**:645–655.

Welburn, J. P. I., M. Vleugel, D. Liu, J. R. Yates, M. A. Lampson, T. Fukagawa, and I. M. Cheeseman (2010). Aurora B phosphorylates spatially distinct targets to differentially regulate the kinetochore-microtubule interface. *Mol. Cell*, **38**:383–392.

Westermann, S., D. G. Drubin, and G. Barnes (2007). Structures and functions of yeast kinetochore complexes. *Annu. Rev. Biochem.*, **76**:563–591.

Widom, J.; Klug, A., Structure of the 300A chromatin filament: X-ray diffraction from oriented samples. *Cell* **1985,** *43* (1), 207-13.

Wieland, G., S. Orthaus, S. Ohndorf, S. Diekmann, and P. Hemmerich (2004). Functional complementation of human centromere protein A (CENP-A) by Cse4p from Saccharomyces cerevisiae. *Mol. Cell. Biol.*, **24**:6620–6630.

Williams, J. S., T. Hayashi, M. Yanagida, and P. Russell (2009). Fission yeast Scm3 mediates stable assembly of Cnp1/CENP-A into centromeric chromatin. *Mol. Cell*, **33**:287–298.

Williams, S. P.; Athey, B. D.; Muglia, L. J.; Schappe, R. S.; Gough, A. H.; Langmore, J. P., Chromatin fibers are left-handed double helices with diameter and mass per unit length that depend on linker length. *Biophys J* **1986,** *49* (1), 233-48.

Winter, S., E. Simboeck, W. Fischle, G. Zupkovitz, I. Dohnal, K. Mechtler, G. Ammerer, and C. Seiser (2008). 14-3-3 Proteins recognize a histone code at histone H3 and are required for transcriptional activation. *EMBO J.*, **27**:88–99.

Witt, O., W. Albig, and D. Doenecke (1996). Testis-specific expression of a novel human H3 histone gene. *Exp. Cell Res.*, **229**:301–306.

Wong, O. K. and G. Fang (2005). Plx1 is the 3F3/2 kinase responsible for targeting spindle checkpoint proteins to kinetochores. *J. Cell Biol.*, **170**:709–719.

Woodcock, C. L. and S. Dimitrov (2001). Higher-order structure of chromatin and chromosomes. *Curr. Opin. Genet. Dev.*, **11**:130–135.

Woodcock, C. L.; Ghosh, R. P., Chromatin higher-order structure and dynamics. *Cold Spring Harb Perspect Biol* **2010,** *2* (5), a000596.

Yan, H., H. Ito, K. Nobuta, S. Ouyang, W. Jin, S. Tian, C. Lu, R. C. Venu, G.-L. Wang, and P. J. Green (2006). Genomic and genetic characterization of rice Cen3 reveals extensive transcription and evolutionary implications of a complex centromere. *Plant Cell*, **18**:2123–2133.

Ye, J.; Ai, X.; Eugeni, E. E.; Zhang, L.; Carpenter, L. R.; Jelinek, M. A.; Freitas, M. A.; Parthun, M. R., Histone H4 lysine 91 acetylation a core domain modification associated with chromatin assembly. *Mol Cell* **2005,** *18* (1), 123-30.

Yuan, J., R. Adamski, and J. Chen (2010). Focus on histone variant H2AX: to be or not to be. *FEBS Lett.*, **584**:3717–3724.

Zalensky, A. O., J. S. Siino, A. A. Gineitis, I. A. Zalenskaya, N. V. Tomilin, P. Yau, and E. M. Bradbury (2002). Human testis/sperm-specific histone H2B (hTSH2B). Molecular cloning and characterization. *J. Biol. Chem.*, **277**:43474–43480.

Zeitlin, S. G., C. M. Barber, C. D. Allis, K. F. Sullivan, and K. Sullivan (2001b). Differential regulation of CENP-A and histone H3 phosphorylation in G2/M. *J. Cell Sci.*, **114**:653–661.

Zeitlin, S. G., N. M. Baker, B. R. Chapados, E. Soutoglou, J. Y. J. Wang, M. W. Berns, and D. W. Cleveland (2009). Double-strand DNA breaks recruit the centromeric histone CENP-A. *Proc. Natl. Acad. Sci. U.S.A.*, **106**:15762–15767.

Zeitlin, S. G., R. D. Shelby, and K. F. Sullivan (2001a). CENP-A is phosphorylated by Aurora B kinase and plays an unexpected role in completion of cytokinesis. *J. Cell Biol.*, **155**:1147–1157.

Zhang, H.; Roberts, D. N.; Cairns, B. R., Genome-wide dynamics of Htz1, a histone H2A variant that poises repressed/basal promoters for activation through histone loss. *Cell* **2005,** *123* (2), 219-31.

Zheng, Y., M. L. Wong, B. Alberts, and T. Mitchison (1995). Nucleation of microtubule assembly by a gamma-tubulin-containing ring complex. *Nature*, **378**:578–583.

Zhou, J., J. Y. Fan, D. Rangasamy, and D. J. Tremethick (2007). The nucleosome surface regulates chromatin compaction and couples it with transcriptional repression. *Nat. Struct. Mol. Biol.*, **14**:1070–1076.

Zhu, C., J. Zhao, M. Bibikova, J. D. Leverson, E. Bossy-Wetzel, J.-B. Fan, R. T. Abraham, and W. Jiang (2005). Functional analysis of human microtubule- based motor proteins, the

kinesins and dyneins, in mitosis/cytokinesis using RNA interference. *Mol. Biol. Cell*, **16**:3187–3199.