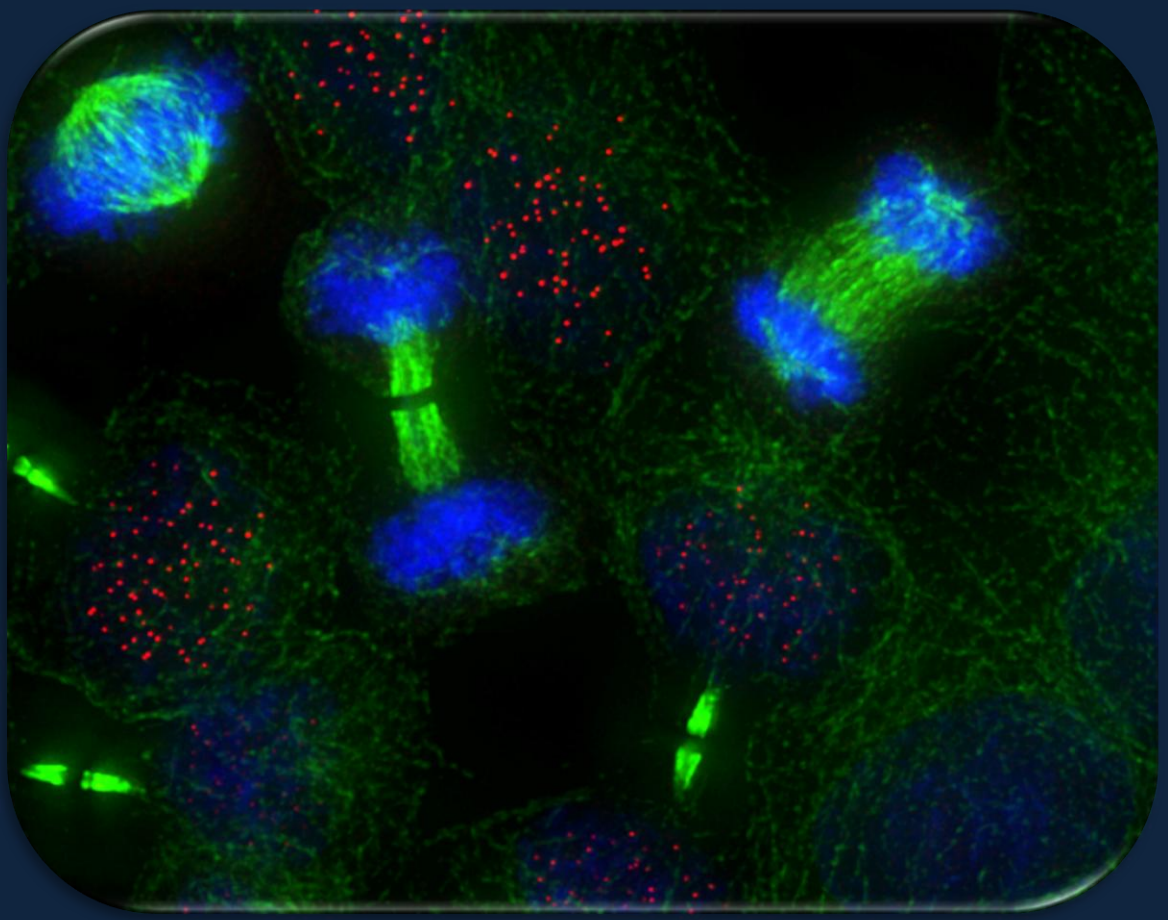
Epigenetic and cell cycle control of centromere inheritance

Mariana Coelho Correia da Silva



Dissertation presented to obtain the Ph.D degree in Cell Biology

Instituto de Tecnologia Química e Biológica | Universidade Nova de Lisboa

Oeiras,
March, 2012

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Declaração

Declaro que esta dissertação de candidatura ao grau de Doutor é da minha autoria e que os dados aqui incluídos são o resultado de trabalho original por mim desenvolvido entre Outubro de 2007 e Outubro de 2011 no laboratório do Dr Lars Jansen, Instituto Gulbenkian de Ciência em Oeiras, Portugal. Este doutoramento foi realizado no âmbito do Programa Doutoral do Instituto Gulbenkian de Ciência PGD 2007. Todas as colaborações estão indicadas em cada capítulo, na secção de Acknowledgements. Esta dissertação teve o apoio financeiro da FCT BD nº SFRH/BD/33219/2007 e dos projectos PTDC/BIA-BCM/100557/2008, Marie Curie Reintegration grant (FP7-PEOPLE-2007-4-3-IRG) e EMBO installation grant.

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Abstract

Cell division is a fundamental process of all living organisms by which a parental cell divides into two genetically identical daughter cells. Faithful cell division requires duplication and subsequent equal distribution of the parental genetic information, the genome, between daughter cells. In eukaryotes, genomic information is organized in chromosomes, which consist of linear DNA sequences packaged into histone protein-DNA complexes called nucleosomes. Chromosomes comprise a defined region, the centromere, which is responsible for delivering the correct number of chromosomal copies to daughter cells during cell division. The centromere directs the formation of the kinetochore, a proteinaceous structure that is responsible for connecting chromosomes to spindle microtubules during mitosis, allowing accurate segregation of chromosomes across generations. Centromere identity in most eukaryotes is not specified by any particular DNA sequence, but rather by an epigenetic chromatin-based mechanism. Key to the epigenetic propagation of the centromere is the histone H3 variant CENP-A that is uniquely incorporated into centromeric chromatin. Consequently, replication and inheritance of this epigenetic mark is crucial to epigenetically maintain centromere identity across cell divisions. Consistently, CENP-A nucleosomes are stably maintained throughout the cell cycle, being turned over only by redistribution between the two sister chromatids during DNA replication. Unlike assembly of the canonical histone H3.1, CENP-A assembly is uncoupled from DNA replication and occurs during late telophase/early G1 in metazoans. Gaining insight into how CENP-A chromatin is propagated throughout cell divisions has been a major research focus in recent years and is essential for our broad understanding of the mechanisms of cell division.

In chapter 2 of this thesis, we describe the identification of new players of the CENP-A assembly pathway, in order to better characterize the molecular

mechanism underlying centromere propagation. Using siRNA-mediated depletion in combination with a unique method to visualize centromere assembly of nascent CENP-A (SNAP-tagging), we show that CENP-N and CENP-C (two proteins that bind directly to CENP-A nucleosomes), and also CENP-T, contribute to CENP-A incorporation into centromeres. This result reveals that these structural components of the centromere are part of an epigenetic feedback loop responsible for propagation of centromeric chromatin.

Although many proteins have been identified to play a role in CENP-A targeting to centromeres, it was still unclear how centromere propagation is restricted to late telophase/early G1 phase. In Chapter 3 we describe the identification of the molecular signal that initiates CENP-A assembly exclusively upon mitotic exit. This phase of the cell cycle is marked by extensive reorganization of the centromere, kinetochore, chromatin, chromosomes and the nucleus as a whole. In addition, a large number of proteins are either selectively destroyed or post-translationally modified at this stage. Using pharmacological and genetic experiments, we show that although CENP-A assembly initiation is not directly dependent on any of these aspects of mitosis, it does require the down regulation of the major mitotic master regulator cyclin-dependent kinase 1 (Cdk1). Additionally, we show that specific inhibition of both Cdk1 and Cdk2 in any phase of the cell cycle is sufficient to trigger rapid CENP-A assembly without passage through mitosis. Neither *de novo* synthesis nor protein destruction are required to trigger CENP-A assembly indicating that the CENP-A assembly machinery is already present in an inactive state prior to mitosis.

In Chapter 4, we sought to characterize the inhibitory mechanism mediated by Cdk activity that regulates the timing of CENP-A assembly and restricts it to late telophase/early G1 phase of the cell cycle. We show that high Cdk1 or Cdk2 activity prevents centromere targeting of well-characterized CENP-A assembly factors such as Mis18α, Mis18BP1^{HsKNL2}, along with the CENP-A specific chaperone HJURP. These proteins are essential for both canonical G1 assembly and unscheduled assembly of CENP-A. We further demonstrate that

Mis18BP1^{HsKNL2} is phosphorylated in a cell cycle dependent manner, and that phosphorylation prevents its centromere localization. In addition, we identify a domain in HJURP, conserved among vertebrates, as a regulatory domain required for timely control of CENP-A assembly. Together these results suggest that Cdk1 and Cdk2 control the cell cycle timing of CENP-A assembly by inhibiting Mis18BP1^{HsKNL2} and potentially other assembly factors through phosphorylation and consequent delocalization.

In conclusion, this work contributes to our understanding of both the epigenetic and cell cycle control mechanisms underlying centromere propagation. We identify novel players in the CENP-A assembly process and unravel the basic mechanism that restricts this assembly to a specific once-per-cell-cycle window, thereby ensuring tight coordination between cell division and epigenetic propagation of the centromere.

Sumário

O processo de divisão celular, pelo qual uma célula mãe se divide para dar origem a duas células filhas, é fundamental para todos os organismos vivos. Para que uma célula se divida correctamente é necessário a duplicação de toda a sua informação genética, que posteriormente é distribuída igualmente por duas células filhas. Nas células eucariotas, a informação genética está organizada em cromossomas. Os cromossomas são constituídos por sequências lineares de ADN (ácido desoxirribonucleico) enroladas em torno de um conjunto de proteínas chamadas histonas. Os complexos formados por histonas e ADN denominam-se de nucleossomas e são as unidades básicas do cromossoma. Os cromossomas possuem uma estrutura designada centrómero, essencial para a distribuição do número correcto de cromossomas pelas células filhas durante o processo de divisão celular. O centrómero é responsável pela formação do cinetocoro, um complexo multiproteico que liga os cromossomas aos microtúbulos do fuso mitótico durante a mitose. Deste modo, o centrómero permite que os cromossomas sejam distribuídos igual e correctamente pelas duas células filhas. Na maioria dos eucariotas, o centrómero é herdado de célula para célula por mecanismos epigenéticos, isto é, independentemente de qualquer sequência especifica de ADN. A proteína CENP-A, uma variante da histona H3, é essencial para a propagação do centrómero. Esta proteína, é incorporada apenas na cromatina centromérica. Logo, a duplicação e herança desta marca epigenética é essencial para a correcta transmissão do centrómero ao longo de diversas gerações. Consistentemente, os nucleossomas que contêm CENP-A são extremamente estáveis, e durante a replicação do ADN são reutilizados e distribuídos pelos dois cromatídeos recentemente formados. Ao contrário do que acontece com a histona H3, a inclusão de CENP-A nos nucleosomas centroméricos ocorre desfasada e independentemente da duplicação do ADN. Em células animais, a incorporação de CENP-A na

cromatina tem início após as células terminarem a mitose e continua durante a fase G1 do ciclo celular. Perceber como a cromatina centromérica é propagada durante a divisão celular é uma área de investigação que tem ganho destaque nos últimos anos e é essencial para o conhecimento geral dos mecanismos da divisão celular.

No capítulo 2 desta tese, descrevemos a identificação de novas proteínas envolvidas no processo de incorporação de CENP-A no centrómero, de modo a perceber detalhadamente o mecanismo molecular responsável pela propagação desta estrutura. Focámos a nossa atenção particularmente em proteínas capazes de se associarem directamente com os nucleossomas de CENP-A. Utilizando uma técnica que permite visualizar apenas a CENP-A recentemente sintetizada e que foi incorporada no centrómero (*SNAP-tagging*) em combinação com a depleção de proteínas utilizando oligos de siRNA, identificámos três novas proteínas que participam no processo de inclusão de CENP-A no centrómero. Com esta metodologia, demonstrámos que duas proteínas que se ligam directamente aos nucleossomas de CENP-A, CENP-C e CENP-N, e também a proteína CENP-T, participam no processo de propagação da cromatina centromérica. A localização destas proteínas no centrómero é, por sua vez, dependente da presença de CENP-A. Este resultado indica que a CENP-A e estes componentes estruturais do centrómero se regulam mutuamente de modo a permitir a transmissão do centrómero através de gerações.

Apesar da recente identificação de várias proteínas que fazem parte do processo de incorporação de CENP-A, permanece por descobrir o mecanismo responsável por limitar a propagação do centrómero até que o processo de mitose seja concluído. No capítulo 3, descrevemos o mecanismo responsável pela activação da transmissão do centrómero após a conclusão do processo de mitose. Esta fase do ciclo celular é caracterizada pela reorganização do centrómero, cinetocoro, cromatina, cromossomas e inclusive de todo o núcleo. Além disso, muitas proteínas são degradadas selectivamente ou são modificadas pós-transcricionalmente durante esta fase. Através do uso de

técnicas genéticas e farmacológicas mostrámos que nenhum destes aspectos da mitose está directamente envolvido na propagação do centrómero. O único factor comum necessário é a inactivação de uma proteína extremamente importante para o controlo da divisão celular, a cinase dependente de ciclina 1 (Cdk1). A inactivação das Cdk1 e Cdk2 em qualquer fase do ciclo celular é suficiente para induzir a incorporação de CENP-A no centrómero sem o envolvimento da mitose. A propagação do centrómero não depende da síntese nem da degradação de proteínas, indicando que as proteínas necessárias para inserir CENP-A na cromatina centromérica estão presentes mas inactivas entre a fase S e a mitose.

No capítulo 4, explorámos o mecanismo de inibição dependente da Cdk1, que controla o *timing* da inclusão da CENP-A no centrómero, e que limita este *timing* ao período final da mitose e à fase G1 do ciclo celular. Mostrámos que a actividade elevada de Cdk1 e Cdk2 impede a localização centromérica de proteínas necessárias à propagação de CENP-A, tais como Mis18α, Mis18BP1^{HsKNL2} e HJURP, a *chaperone* específica da CENP-A. Estas proteínas são essenciais tanto para a propagação natural do centrómero na fase G1 como para a propagação induzida nas fases anteriores à mitose. Mostrámos também que Mis18BP1^{HsKNL2} é fosforilada em períodos específicos do ciclo celular e que esta fosforilação impede a localização desta proteína no centrómero. Adicionalmente, identificámos um domínio regulatório na proteína HJURP, necessário para controlar o *timing* de propagação de CENP-A. De notar que este domínio é conservado em todos os vertebrados. Em conjunto, estes resultados indicam que Cdk1 e Cdk2 controlam o *timing* da transmissão do centrómero através da inactivação da Mis18BP1^{HsKNL2} e potencialmente de outras proteínas. Esta inactivação depende da fosforilação destas proteínas e, consequente, da sua deslocalização.

Resumidamente, identificámos novos participantes do processo de propagação de CENP-A e revelámos o mecanismo que limita a propagação do centrómero a um único período do ciclo celular, assegurando a coordenação

entre o processo de divisão celular e a herança epigenética do centrómero. Deste modo, este trabalho contribui para o conhecimento dos mecanismos de controlo epigenético e temporal responsáveis pela propagação do centrómero.

List of Abbreviations

(A) Ala	Alanine
APC/C	Anaphase Promoting Complex/Cyclosome
ATP	Adenosine triphosphate
Borax	Tetraborato de sódio ($Na_2B_4O_7 \cdot 10H_2O$)
bp	Base pair
BrdU	Bromodeoxyuridine
BTP	Bromothenylpteridine
CAF1	Chromatin Assembly Factor 1
CAK	Cdk-Activating Kinase
CATD	CENP-A Targeting Domain
CCAN	Constitutive Centromere-Associated Network
Cdc	Cell Division Cycle
Cdc20	Cell Division Cycle 20 homologue
CDE	Centromere DNA Element
Cdh1	Cdc20 Homologue 1
Cdk	Cyclin-Dependent Kinase
cDNA	Complementary DNA
Cdt1	Chromatin licensing and DNA replication factor 1
CENP	Centromere Protein
CENP-A	Centromere Protein A
ChIP	Chromatin Immunoprecititation
CKI	Cdk inhibitor
CMG	Cdc45-Mcm2-7-GINS
DAPI	4',6-diamidino-2-phenylindole
DNMT	DNA Methyltransferase
DNA	Deoxyribonucleic acid
DT40	Chicken B cell line

E (Glu)	Glutamate
Emi1	Early Mitotic Inhibitor
FACS	Fluorescence-Activated Cell Sorting
FBS	Fetal Bovine Serum
FRAP	Fluorescence Recovery After Photobleaching
G (Gly)	Glycine
GBP	GFP binding protein
GEF	Nucleotide Exchange Factor
GFP	Gree Fluorescent Protein
HA	Hemagglutinin epitope tag
HCl	Hydrochloric acid
HDAC	Histone Deacetylase
HeLa	Henrietta Lacks Cervical Cancer Cell Line
HFD	Histone Fold Domain
HIRA	Hir-related protein A
HJURP	Holliday Junction Recognizing Protein
HP1	Heterochromatin-Associated Protein 1
HRP	Horseradish Peroxidase
hTERT	Human Telomerase Reverse Transcriptase
HU	Hydroxyurea
ICEN	Interphase Centromere Complex
K (Lys)	Lysine
kb	kilo bases
KMT	Lysine Methyltransferase
KNL2	Kinetochore Null 2
L (Leu)	Leucine
LINE	Long Interspersed Elements
MAP	Microtubule-Associated Protein
Mb	Mega bases

MCC	Mitotic Checkpoint Complex
MCM	Minichromosome Maintenece Complex
M	Methionine
Mis	*S. pombe* mutants with high loss rate of minichromosomes
Mis18BP1	Mis18 Binding Protein 1
mRNA	Messenger RNA
MTOC	Microtubule Organizing Center
NaV	Sodium Orthovanadate
NEBD	Nuclear Envelope Breadown
NPM/B23	Nucleophosmin
ORC	Origin Recognition Complex
ORF	Open Reading Frame
P (Pro)	Proline
PC	Polycomb protein
PcG	Polycomb Group
PCM	Pericentriolar material
PCNA	Proliferating Cell Nuclear Agent
PCR	Polymerase Chain Reaction
Plk1	Polo Like Kinase 1
PP1	Protein phosphatase 1
PP2A	Protein phosphatase 2A
pRb	Retinoblastoma protein
PRC	Polycomb Repressive Complex
pre-RC	Pre-Replicative Complex
R (Arg)	Arginine
RbAp46	Retinoblastoma-associated protein 46kDa
RbAp48	Retinoblastoma-associated protein 48kDa
RNAi	RNA interference

RPE	Retinal Pigment Epithelial Cell Line
RSF	Remodeling and Spacing Factor
RT	Room Temperature
SCF	Skp1/Cul1/F-box
S (Ser)	Serine
SINE	Short Interspersed Elements
T (Thr)	Treonine
TMR	Tetramethylrhodamine
Tome-1	Trigger of Mitotic Entry 1
TrxG	Trithorax Group
TSA	Trichostatin A
Ub	Ubiquitin
WT	Wild Type

Table of Contents

Chapter 1 – General Introduction

1. Chromosome structure and genetic inheritance

1.1. Packing of DNA into chromosomes

DNA is the primary carrier of genetic information in nearly all living organisms. In eukaryotes, the total length of DNA sequences can reach up to a hundred thousand times the diameter of the cell. Therefore, compartmentalization of the genetic material inside the nucleus of the cell requires a dramatic packing of DNA molecules into a protein-DNA complex called chromatin in eukaryotes. Chromatin is primarily composed of double stranded DNA folded around small basic proteins called histones (Kornberg, 1974). The fundamental repeating unit of chromatin is the nucleosome (Oudet et al., 1975). Each nucleosome is comprised of a DNA segment with 146 base pairs (bp) in length wrapped around a compact histone protein core (Luger et al., 1997). This histone core is composed of an octamer containing two copies of each of the canonical histones H2A, H2B, H3, and H4 (Richmond et al., 1984; Luger et al., 1997). The structure of histones is strongly conserved across evolution, suggesting that this type of DNA packaging has evolved very early and is one of the defining features of eukaryotes.

The packing of DNA into nucleosomes shortens the DNA length about sevenfold. Further compaction is achieved by folding of nucleosomes into higher order arrays with multiple levels of packing ultimately resulting in mitotic chromosomes. The nature of these higher order structures is a matter of debate but may include the formation of a fiber about 30 nm wide, whose formation is facilitated by the linker histone H1 [Figure 1.1; (Everid et al., 1970; Thoma et al., 1979)]. Additional levels of compaction are achieved by looping the chromatin fibers along a structural scaffold formed by non-histone proteins such as topoisomerase II and the condensin complex [Figure 1.1; (Moser and Swedlow, 2011)]. A higher degree of compaction is still achieved during mitosis, the

process by which a cell separates its chromosomes into two identical sets and distributes them between two daughter cells.

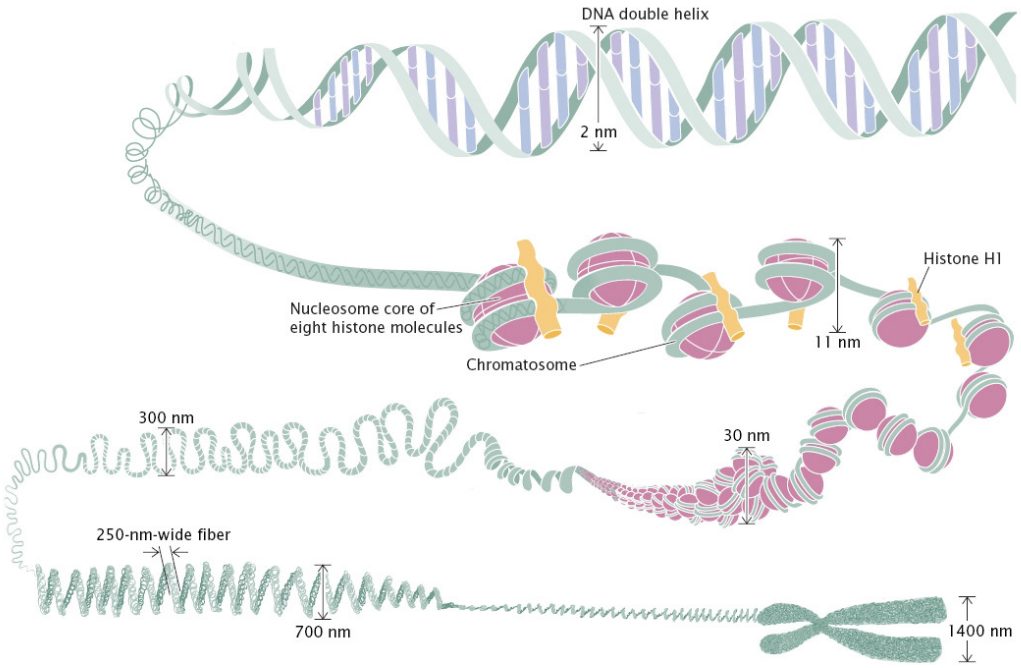

Figure 1.1. Model for the packing of chromatin into metaphase chromosomes. The simplest level of organization of the genetic material is the double stranded helical structure of DNA. DNA is folded around specialized eukaryotic proteins called histones to form nucleosomes, the primary repeating unit of chromatin. Each nucleosome consists of a core of eight histone proteins around which the DNA wraps 1,65 times. This first level of compaction is facilitated by the linker histone H1 that binds to each nucleosome forming the chromatosome. Nucleosomes further fold around each other to produce a fiber about 30 nm wide that forms loops averaging 300 nm in length. The 300 nm fibers are compressed and folded to create a fiber with a width of 250 nm. Tight coiling of these fibers is responsible for the final level of chromatin compaction into a metaphase chromosome. Adapted from Pierce, 2005.

The multiple packing levels of chromatin serve not only as a way to compact the DNA within the eukaryotic nucleus, but also play important functional roles. Chromatin packing offers distinct mechanisms for controlling gene

expression and DNA metabolism, principally by controlling access of regulatory proteins to DNA. Highly compacted chromatin is not accessible to the enzymes involved in DNA transcription, replication or repair. Therefore, regions of chromatin where active transcription takes place (euchromatin) are less condensed than regions where transcription is inactive or is actively repressed (constitutive and facultative heterochromatin, respectively) (Horn and Peterson, 2002; de la Serna and Imbalzano, 2002). Alternatively, looping of nucleosome-containing fibers can bring specific regions of chromatin together, thereby influencing gene expression (Misteli, 2007).

The state of chromatin can be regulated directly by energy consuming chromatin remodeling motors that change chromatin conformation, allowing targeted access of regulatory proteins, for example, to specific genes (Varga-Weisz and Becker, 2006). Additionally, gene expression is controlled by modification of histones with small chemical moieties, such as methyl and acetyl groups on the N-terminal tail that extend from the core particle of the nucleosomes (Jenuwein and Allis, 2001; Wang et al., 2004b). The importance of these histone modifications will be further discussed in the context of centromere function and epigenetics in the following sections.

1.2. Genetic inheritance across cell divisions

The faithful inheritance of genetic information during meiosis and mitosis is central to the growth and development of all living organisms. Aberrant chromosome inheritance causes the formation of cells with an abnormal number of chromosomes, called aneuploid cells (Torres et al., 2008). Aneuploid cells are unstable and are normally eliminated by apoptosis. However, in rare cases, aneuploidy can result in tumor formation and in the development of severe genetic disorders associated with birth defects (Hassold and Hunt, 2001; Kops et al., 2005; Weaver et al., 2007).

The universal process responsible for the formation of two genetically identical cells is called the cell cycle. The cell cycle can be divided in four stages. The two critical phases are S phase (synthesis phase; during which the genetic material is duplicated) and M phase (mitosis; during which the duplicated material is distributed between the two daughter cells). These two phases are separated by two gap phases: G1 phase (first gap phase; preceding S phase) and G2 phase (second gap phase; preceding mitosis) (reviewed in Nurse, 2000; Pollard et al., 2004). G1, S, and G2 phases are collectively called interphase, the period between one mitosis and the next (Figure 1.2). Not only the chromosomes, but also the cytoplasm and the cell organelles are divided between two daughter cells during mitosis and subsequent cytokinesis.

Mitosis is a complex process that is divided into five discrete cell biologically defined stages: prophase, prometaphase, metaphase, anaphase and telophase (Figure 1.2). During prophase chromosomes condense and centrosomes, the organelles that serve as the main microtubule organizing centers (MTOC) in animal cells, migrate to opposite poles of the cell to facilitate the assembly of the mitotic spindle (Figure 1.2). At the end of prophase, nuclear envelope breakdown (NEBD) occurs and cells enter prometaphase. During prometaphase the formation of the mitotic spindle is completed and chromosomes start attaching to spindle microtubules (Figure 1.2). The chromosome-microtubule attachment is mediated by a chromosome-borne multiprotein complex called the kinetochore. Once all kinetochores are attached to microtubules that emanate from opposite poles, the chromosomes move to the middle of the cell. When all chromosomes are properly attached and aligned, the cell reaches metaphase (Figure 1.2). During anaphase, the chromosomes are separated into two sister chromatids that migrate to opposite poles of the spindle (Figure 1.2). When the chromatids approach the spindle poles and nuclear envelope reformation starts, the cell is in telophase (Figure 1.2). At the end of telophase, a contractile actin-myosin ring is formed between the chromatin

masses, leading to separation of the two daughter cells (cytokinesis) (Morgan, 2007).

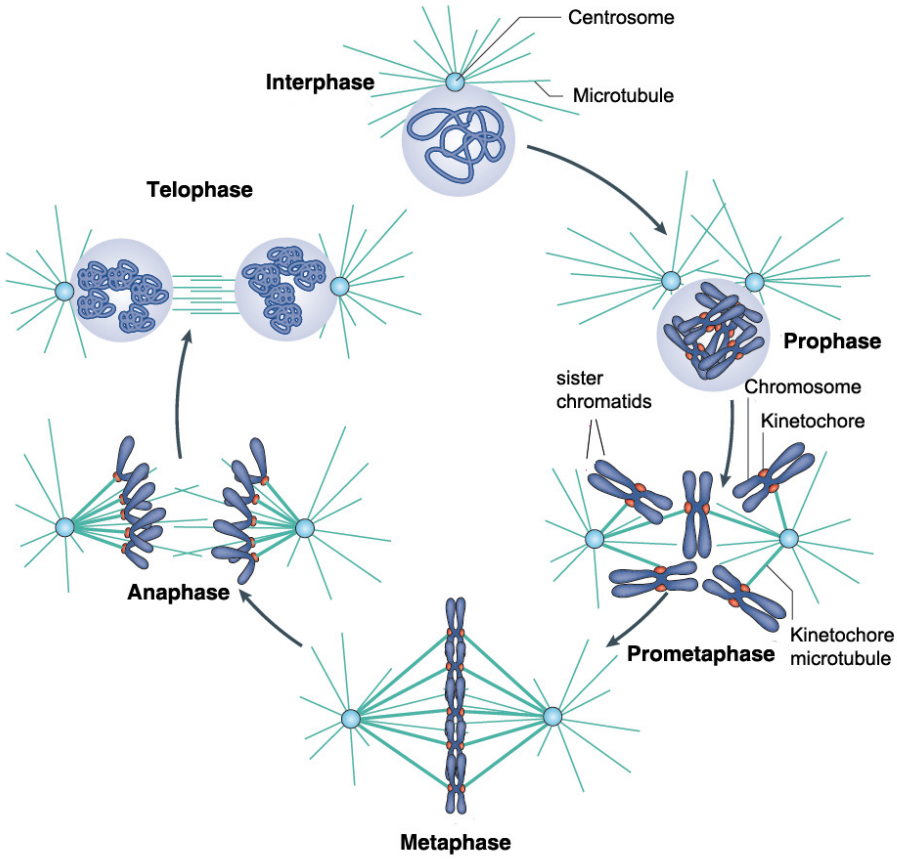

Figure 1.2. The stages of mitosis in animal cells. The various stages of the cell cycle are depicted. During interphase the cell undergoes growth (G1 phase) and replication of the DNA (S phase). Upon duplication of the centrosomes and DNA, the cell undergoes a second round of growth (G2 phase) and subsequently enters mitosis. Mitosis can be divided in five stages: prophase, prometaphase, metaphase, anaphase and telophase. See the text for details. Adapted from Verdaasdonk and Bloom, 2011.

Meiosis occurs only in germ cells and is characterized by a single round of DNA replication followed by two divisions called meiosis I and meiosis II. Unlike mitosis that leads to the formation of two diploid daughter cells, meiosis produces gametes: haploid eggs in the female germ line and haploid sperm in the male

germ line. In meiosis I, the replicated homologous chromosomes are segregated to opposite poles, and in meiosis II, the sister chromatids of each homolog are segregated (Kleckner, 1996).

1.3. Chromosomal elements required for proper genetic inheritance

Accurate genome duplication and inheritance during mitosis and meiosis require specific chromosomal elements and protein complexes (Allis et al., 2007). These chromosomal elements include replication origins, centromeres and telomeres (Figure 1.3).

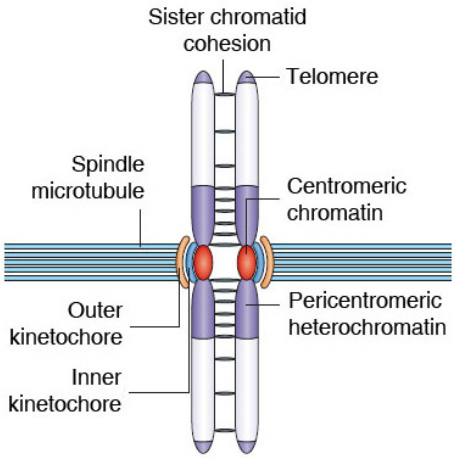

Figure 1.3. Key elements of chromosome inheritance. The diagram indicates chromosomal elements or protein complexes essential for proper inheritance of the genetic material: the centromere, kinetochores, telomeres and sister chromatin cohesion. Centromeres include centromeric chromatin and pericentromeric heterochromatin. Centromeric chromatin nucleates the formation of the kinetochore, which forms the site of attachment for spindle microtubules. Pericentric heterochromatin is the site of cohesion accumulation, contributing to sister chromatid cohesion. Telomeres form the ends of chromosomes, protecting them from degradation and fusion. Adapted from Allshire and Karpen, 2008.

Replication origins are the defined chromosomal regions where DNA replication is initiated in a eukaryotic cell. At each origin, two replication forks are formed, and replication proceeds bidirectionally until the replication forks encounter another fork approaching from the opposite direction. The mechanism responsible for the formation and regulation of replication origins will be discussed in Section 4.1.4 of this Chapter.

Several important processes occur at the replication fork, such as assembly of new histones to form chromatin. Proteins required for sister chromatid cohesion, called cohesins, are also assembled during this stage, but associate with chromosomes before DNA replication. In this way, sister chromatid cohesion is ensured to maintain duplicated sister chromatids together until anaphase onset (Nasmyth and Haering, 2009).

While cohesion holds newly replicated sister chromatids together, centromeres are chromosomal elements crucial to drive separation of chromosomes during mitosis. The centromere is a chromosomal locus composed of DNA and specialized chromatin proteins that serve as the foundation for kinetochore formation during mitosis (Allshire and Karpen, 2008; Silva and Jansen, 2009). The kinetochore is a multi-protein complex that links each chromosome to spindle microtubules, ensuring chromosome movement and proper chromosome segregation during mitosis and meiosis (Cleveland et al., 2003; Cheeseman and Desai, 2008). The detailed function and structure of centromeres will be discussed in the Section 2 of this Chapter.

Finally, telomeres are important chromosome elements that are located at the end of the chromosomes to protect them from degradation and to prevent chromosome rearrangements, such as chromosome fusion (O'Sullivan and Karlseder, 2010).

Defects in sister chromatid cohesion or loss of telomere and/or centromere functions result in chromosome instability, which can cause or contribute to the development of tumors (Bailey and Murnane, 2006; Ricke et al., 2008; Thompson et al., 2010).

2. Organization and function of centromeres and kinetochores

The centromere is a specialized chromosomal region that was originally identified cytologically as the primary constriction of a metaphase chromosome [Figure 1.4 A; (Fawcett, 1994)]. The broader centromere region serves two purposes. The core region nucleates the kinetochore, which in turn ensures proper chromosome segregation during mitosis and meiosis (Cleveland et al., 2003). A second centromeric domain that surrounds the kinetochore named pericentromeric heterochromatin is the site of cohesion accumulation during mitosis and thereby contributes to sister chromatid cohesion until anaphase onset (Sullivan, 2001; Cleveland et al., 2003; Ekwall, 2007).

Based on the size and localization of the centromere, eukaryotic chromosomes can be classified into two distinct types: monocentric and holocentric (Figure 1.4 C). Monocentric chromosomes assemble the centromere and kinetochore at a single defined region. In contrast, holocentric chromosomes have “diffuse” centromeres and kinetochores that are formed along the entire length of each chromosome. Although most eukaryotes have monocentric chromosomes (Ekwall, 2007), holocentric chromosomes are found in a wide variety of species such as in certain plants and in various types of animals including nematodes, arachnids, and insects (Schvarzstein et al., 2010). Interestingly, some organisms such as centipedes appear to have both holocentric and monocentric chromosomes in the same nucleus (White, 1973). Among common model organisms, only the nematode *Caenorhabditis elegans* (*C. elegans*) features holocentric chromosomes (Maddox et al., 2004). Localized centromeres present on monocentric chromosomes can be divided in two classes: point centromeres and regional centromeres (Figure 1.4 C). Point centromeres can be found in the budding yeast (*Saccharomyces cerevisiae*), are located on a small stretch of centromeric DNA, and direct the formation of kinetochores that bind to only one microtubule (Cheeseman et al., 2002).

Regional centromeres can be found in several model organisms, such as in the fission yeast (*Schizosaccharomyces pombe*), the fruit fly (*Drosophila melanogaster*), the African clawed frog (*Xenopus laevis*), the chicken (*Gallus gallus),* the mouse *(Mus musculus)* as well as in humans (*Homo sapiens*). Regional centromeres drive the assembly of larger kinetochores on bigger and more complex chromosomal regions and have multiple microtubule attachment sites. How and why such an essential chromosomal component has evolved into markedly different structures is unclear. However, the functional mechanisms involved in the formation and molecular composition of different centromeres are conserved between even the most distant centromere types (Sullivan et al., 2001; Allshire and Karpen, 2008; Joglekar et al., 2008).

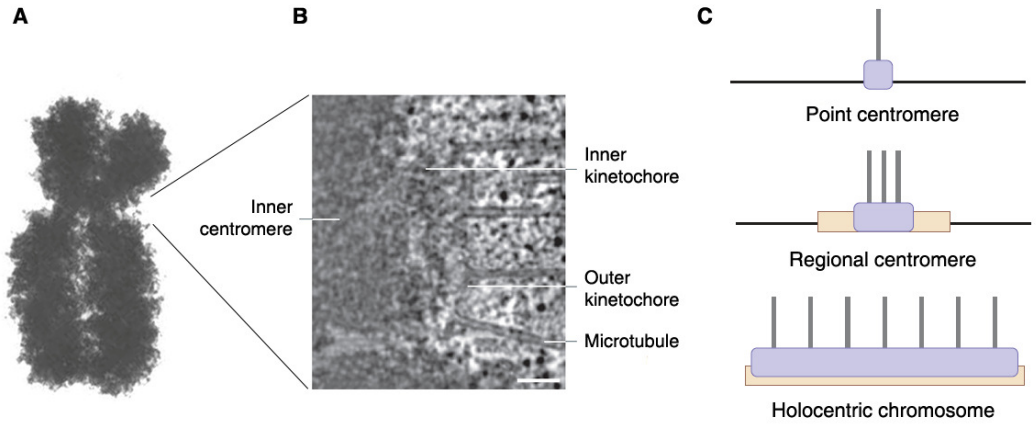

Figure 1.4. Centromere/kinetochore structure. (A) Electron micrograph of an entire chromosome. Adapted from Fawcett, 1994. (B) Electron micrograph of a human kinetochore. The micrograph represents a single slice from a tomographic volume of a high-pressure frozen mitotic cell. The key structural features of the centromere/kinetochore are labelled. Scale bar, 100 nm. Adapted from Cheeseman and Desai, 2008. (C) Schematic of the different types of chromosomes: monocentric, which can contain point or regional centromeres; and holocentric, in which the centromere occupies the entire chromosome. Black lines represent DNA, gray lines represent kinetochore microtubules, beige rectangles represent centromeric DNA, and purple rectangles represent kinetochores. Adapted from Ekwall, 2007.

2.1. Centromeric DNA

Centromeres are directly associated with chromosomal DNA. Therefore, initial models proposed that centromere location and function is directed by specific DNA sequences underlying centromeric chromatin. This occurs in simple eukaryotic organisms such as *S. cerevisiae*. The point centromere of *S. cerevisiae* is defined by a specific DNA sequence found on all chromosomes (Clarke and Carbon, 1983). This characteristic sequence is comprised of three functional elements termed centromere DNA element I (CDEI), CDEII and CDEIII (Figure 1.5 A). Together they form a sequence of approximately 125 bp that is sufficient to confer mitotic stability when introduced into plasmids (Clarke and Carbon, 1980; Fitzgerald-Hayes et al., 1982; Hieter et al., 1985). The sequences of CDEI and CDEIII are conserved among all *S. cerevisiae* chromosomes and are responsible for the recruitment of centromere proteins and kinetochore formation (Mellor et al., 1990; Lechner and Carbon, 1991). CDEII is a 78-86 AT-rich region that organizes a single centromeric nucleosome, which contains a specific histone H3 variant called Cse4 (the *S. cerevisiae* homologue of CENP-A, called CENP-A^{Cse4} throughout this thesis) (Stoler et al., 1995; Meluh et al., 1998).

In fission yeast and metazoans, specific DNA sequences that drive centromere assembly have not been identified. Instead, the centromere in these organisms is formed within highly repetitive tandem sequence repeats (Figure 1.5 B-D) (Tyler-Smith and Floridia, 2000; Choo, 2001). A comparison of these repetitive sequences from different species reveals that centromeric DNA sequences are extremely divergent among eukaryotes. Consistently, centromeric DNA was identified as one of the fastest evolving regions in the genome (Henikoff et al., 2001). In fission yeast, centromeric DNA ranges from 30 to 110 kb in length and contains a central core element that is flanked by various inverted repeats (Figure 1.5 B) (Pidoux and Allshire, 2004). In multicellular eukaryotes, centromeres are composed of repetitive DNA sequences that are organized in long head-to-tail tandem arrays (Figure 1.5 C and D). The

monomeric DNA elements that assemble into these repetitive arrays range in size from 5 or 7 bp (in flies) to 340 bp (in pigs).

In humans, the repeat unit is a 171 bp monomer known as α-satellite (or alphoid) DNA (Willard, 1985, 1990). The arrays formed by α-satellite repeats can range from less than 100 kb up to several megabases (Willard, 1998). Alphoid DNA repeats are present in two distinct subtypes, type I and type II. Type I repeats, also called α-I satellite DNA contain a 17 bp sequence termed the CENP-B box that recruits the conserved centromere protein B (CENP-B) (Earnshaw et al., 1987; Masumoto et al., 1989; Ikeno et al., 1994). α-I satellite repeats are flanked on both sides by divergent repetitive sequences and retrotransposons, which are referred as α-II satellite DNA. These type II satellite repeats are generally interspersed with other repetitive elements such as long and short interspersed elements (LINEs and SINEs, respectively).

Despite significant differences in size and DNA sequence, general features of centromeres across various eukaryotes appear to be highly conserved (Figure 1.5). In general, centromeres are surrounded by heterochromatin regions, are embedded into AT-rich repetitive sequences, and have a similar protein composition (Sullivan and Karpen, 2001; Morris and Moazed, 2007). Therefore, it is likely that, in most eukaryotes, centromere identity and function is mediated by these common features of centromeric chromatin discussed further below.

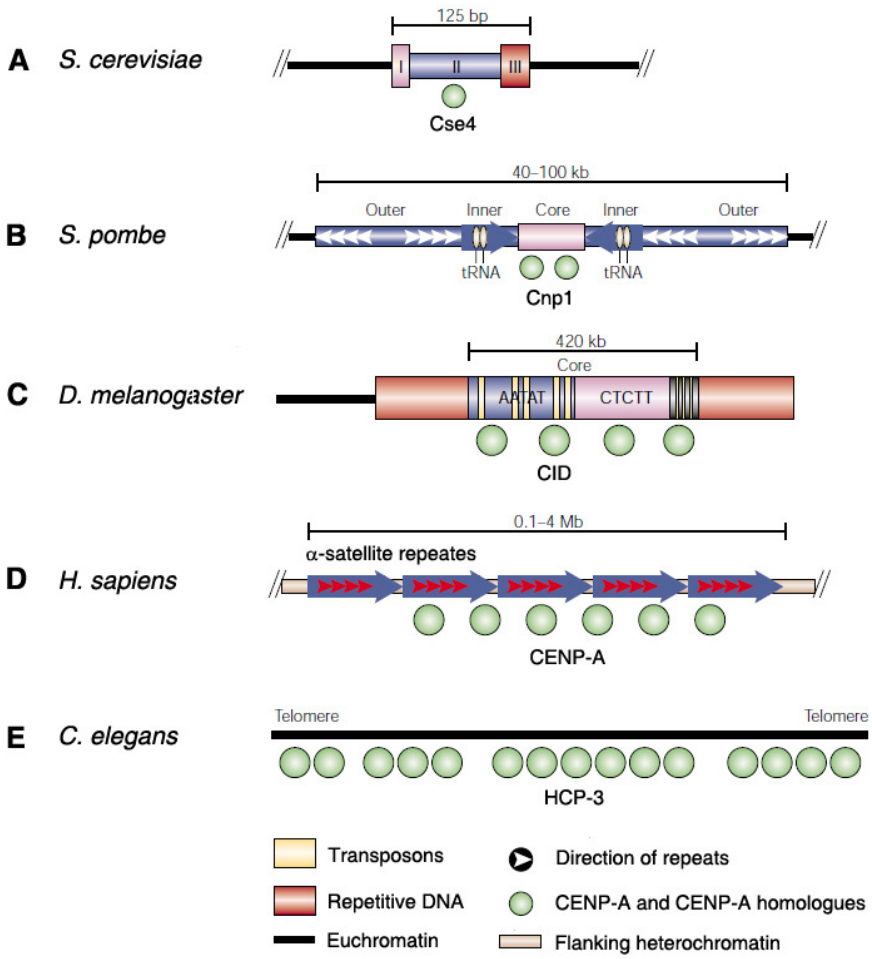

Figure 1.5. Organization of centromeric chromatin in different eukaryotes. The DNA sequence of centromeres differs between species, but the presence and function of CENP-A and its homologues (shown in green) at centromeres is highly conserved. (A) *S. cerevisiae* centromere function depends on conserved DNA elements (I, II, III), to which Cse4 localizes. (B) *S. pombe* centromeres contain a unique central core, the site of Cnp1 nucleosome assembly, flanked by conserved inverted inner and outer repeats. (C) The defined *D. melanogaster* centromere at minichromosome *Dp1187* consists of a core of 5 bp satellites and transposons, flanked by other repetitive DNA (red). (D) Human centromeres consist of α-satellite DNA (red arrows) tandemly arranged into higher order repeats (blue arrows), which extend over megabases. CENPA localizes to a portion of these arrays. (E) *C. elegans* centromeres assemble along the length of each chromosome. Adapted from Sullivan et al., 2001.

2.2. Centromeric chromatin organization

2.2.1. CENP-A, a histone H3 variant unique to the centromere

Histone modifications and histone variants serve to define unique chromosome regions, including the centromere. The centromere differs from the rest of chromatin primarily by the presence of a specific histone H3 variant, the centromere protein A (CENP-A), that replaces canonical histone H3.1 in nucleosomes at active centromeres [Figure 1.6; (Palmer et al., 1987, 1991; Yoda et al., 2000)]. This protein was one of the first centromere proteins identified as an antigen recognized by sera from patients suffering from scleroderma spectrum disease (Earnshaw and Rothfield, 1985; Valdivia and Brinkley, 1985). Homologs of human CENP-A have been identified in all eukaryotes thus far: mouse (Cenpa), chicken (CENP-A), plants (CenH3), *D. melanogaster* (CID), *X. laevis* (CENP-A), *C. elegans* (HCP-3), *S. pombe* (Cnp1) and *S. cerevisiae* (Cse4) (Wulf and Earnshaw, 2008; Silva and Jansen, 2009).

At point centromeres, such as those in *S. cerevisiae*, a single CENP-A^{Cse4}-containing nucleosome forms the basis for kinetochore formation and microtubule attachment (Furuyama and Biggins, 2007). Regional centromeres have multiple CENP-A nucleosomes interspersed between nucleosomes containing the canonical histone H3.1 and nucleosomes containing H3.3, another histone H3 variant [Figure 1.6; (Blower et al., 2002; Dunleavy et al., 2011)]. In holocentric chromosomes, CENP-A and canonical H3 nucleosomes are interspersed and spread throughout chromosome arms (Buchwitz et al., 1999; Oegema et al., 2001). Importantly, loss of CENP-A or CENP-A homologs results in a complete failure in chromosome segregation in all organisms tested so far (Earnshaw and Migeon, 1985; Palmer et al., 1987; Meluh et al., 1998; Buchwitz et al., 1999; Howman et al., 2000; Takahashi et al., 2000; Blower and Karpen, 2001; Oegema et al., 2001; Régnier et al., 2005). This can be explained by the fact that CENP-A is responsible for nucleating the centromere/kinetochore complex, which is central for proper chromosome movement and segregation (Régnier et al., 2005;

Foltz et al., 2006; Liu et al., 2006). Loss of CENP-A results in concomitant loss of several centromere/kinetochore proteins, whereas overexpression of CENP-A^{CID}, in flies, results in mislocalization of CENP-A^{CID} to noncentromeric regions and in the formation of ectopic kinetochores (Heun et al., 2006; Olszak et al., 2011). These observations suggest that CENP-A forms the foundation for kinetochore assembly.

CENP-A and H3 share sequence homology within their histone fold domains (approximately 60% in humans), but there is no sequence identity between N-termini of both proteins (Sullivan et al., 1994). Consistent with the pattern of sequence homology between CENP-A and H3, the histone fold domain (HFD) directs CENP-A deposition at centromeres. A stretch of residues responsible for CENP-A localization are found across the loop L1 and the adjacent α2-helix of the HFD and is called the CENP-A targeting domain (CATD) (Sullivan et al., 1994; Shelby et al., 1997; Vermaak et al., 2002; Black et al., 2004). Replacement of the corresponding region within the HFD of H3 by the CATD creates a chimeric $H3^{CATD}$ protein that targets to centromeres (Black et al., 2004, 2007b). Remarkably, this chimeric histone $H3^{CATD}$ rescues the lethality induced by depletion of CENP-A and sustains the assembly of a functional kinetochore (Black et al., 2007b). Using hydrogen/deuterium exchange experiments, the CATD was found to induce conformational rigidity to (CENP-A/H4)$_2$ tetramers and CENP-A containing nucleosomes relative to the conventional counterparts containing histone H3.1 (Black et al., 2004, 2007a). The molecular basis for this conformational rigidity was later provided by the atomic structure of the subnucleosomal (CENP-A/H4)$_2$ tetramers (Sekulic et al., 2010). The crystal structure of the entire human CENP-A-containing nucleosome has only been recently reported (Tachiwana et al., 2011). Overall, the structure of the CENP-A nucleosome is extremely similar to the structure of canonical H3.1 nucleosome. The major structural differences of CENP-A nucleosomes are: 1) the extended loop L1 that appears at the surface of the nucleosome and could serve as a CENP-A specific contact site, and 2) a wider angle at which DNA

enters and exits the nucleosome resulting in a smaller DNA protection footprint in nuclease assays (Maddox et al., 2011; Tachiwana et al., 2011). These different DNA angles may affect higher order packing of CENP-A nucleosomes that could contribute to maintaining a unique centromeric chromatin structure. Despite these differences, these results indicate that human CENP-A-containing nucleosomes are octameric and contain two copies of each of CENP-A, H4, H2A and H2B. However, we cannot exclude that in other organisms or in different cell cycle stages, CENP-A nucleosomes have alternative structures, such as tetrasomes, hemisomes, and hexasomes, as several reports have suggested (Dalal et al., 2007a, 2007b; Mizuguchi et al., 2007; Furuyama and Henikoff, 2009; Williams et al., 2009).

2.2.2. Patterns of histone modifications at the centromere

The highly homogenous, repetitive nature of centromeric DNA makes evaluation of the long-range chromatin organization of centromeric regions, for example by chromatin immunoprecipitation (ChIP), a challenging task (Spence et al., 2002; Lam et al., 2006). Nevertheless, high resolution cytogenetic techniques such as chromatin fiber analysis have revealed that centromeric chromatin in humans and flies is composed of CENP-A nucleosomes interspersed with nucleosomes containing the canonical H3 dimethylated at Lysine 4 (H3K4me2) [Figure 1.6; (Blower et al., 2002; Sullivan and Karpen, 2004)]. This modification, which is a mark of euchromatin (Bernstein et al., 2002; Schneider et al., 2004), is thought to be important for structural organization of the centromere (Dunleavy et al., 2005). HJURP (Holliday Junction Recognizing Protein) is a CENP-A specific chaperone important for CENP-A assembly (Dunleavy et al., 2009; Foltz et al., 2009). Importantly, loss of H3K4me2 results in failure of centromere targeting of HJURP, impairing incorporation of CENP-A at centromeres (Bergmann et al., 2011).

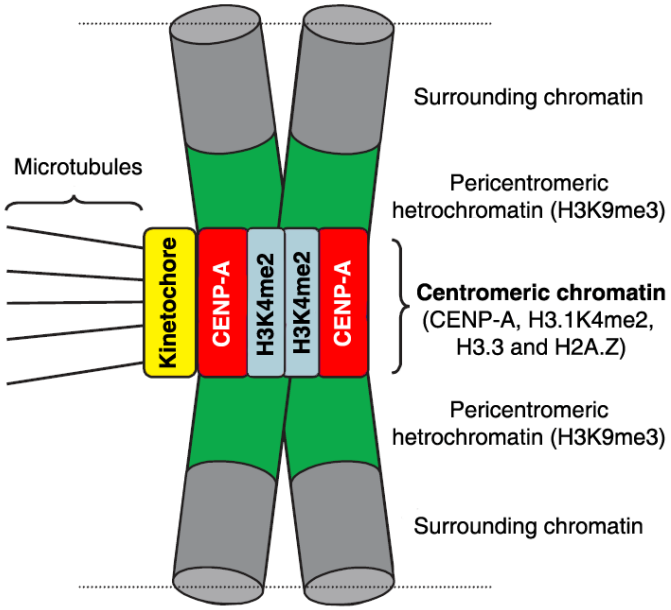

Figure 1.6. The unique organization of centromeric chromatin and pericentromeric heterochromatin. Centromeric chromatin consists of CENP-A nucleosomes interspersed with histone H3 nucleosomes. These H3 nucleosomes feature a unique pattern of histone modifications rich in H3K4me2 and low in acetylation as well as the histone variant H2A.Z. Centromeric chromatin also features nucleosomes containing H3.3, which may function as a CENP-A placeholder during S, G2 and M phases. In metaphase chromosomes, it has been proposed that these different histone variants are organized in distinct domains (represented in red and blue). The CENP-A domain is located at the exterior surface, on top of which the kinetochore is formed. The other histones are mainly located at the inner centromere. However, it is possible that H3.1 and H3.3 are also part of the kinetochore forming domain that is in contact with other centromere and kinetochore proteins. Recently a new nucleosome like complex (CENP-T-W-S-X) was identified that is present within the CENP-A containing chromatin domain (see Figure 1.7). Adapted from Vos et al., 2006.

CENP-A/H3K4me2 containing chromatin is typically embedded within a large domain of heterochromatin named pericentromeric heterochromatin [Figure 1.6; (Carroll and Straight, 2006; Verdaasdonk and Bloom, 2011)]. The assembly and inheritance of pericentromeric heterochromatin is thought to be dependent, in

part, on the trimethylation of histone H3 on Lysine 9 (H3K9me3) and on the binding of this histone modification by the chromodomain-containing protein HP1. H3K9me3 chromatin in turn recruits cohesin that is responsible for establishment of sister chromatid cohesion (Nonaka et al., 2002). Indeed, cells that lack Su(var)3-9, the methyltransferase generating the H3K9me3 mark, do not recruit HP1 to pericentromeric heterochromatin and do not establish sister chromatid cohesion (Guenatri et al., 2004). In some species, such as in fission yeast, the RNA interference (RNAi) machinery also plays a critical role in maintaining the pericentromeric heterochromatin (Grewal and Moazed, 2003).

Another feature of centromeric chromatin is the lack of histone acetylation that is normally associated with actively transcribed chromatin. The hypoacetylation of histones at the centromere alter the chromatin structure and organization, defining a chromatin region distinct from traditional euchromatin and heterochromatin (Sullivan and Karpen, 2004; Dunleavy et al., 2005). Combined, these modifications may help maintain the identity of centromeric chromatin and pericentromeric heterochromatin, which are required for kinetochore assembly, sister chromatid cohesion and condensation, and for proper chromosome segregation during mitosis (Hendzel et al., 1997; Bernard et al., 2001).

2.3. The Constitutive Centromere-Associated Network (CCAN)

The core centromere is composed of several proteins that are associated with CENP-A chromatin throughout the cell cycle. Although many designations exist in the literature, this protein complex is now commonly called the constitutive centromere-associated network (CCAN) and is composed of 16 proteins: CENP-C, CENP-H, CENP-I, CENP-K through CENP-U, CENP-W and CENP-X (Obuse et al., 2004; Foltz et al., 2006; Izuta et al., 2006; Okada et al., 2006; Cheeseman and Desai, 2008; Hori et al., 2008; Amano et al., 2009; McAinsh and Meraldi, 2011; Perpelescu and Fukagawa, 2011). Many of these proteins are conserved in vertebrates and fission yeast but, with the exception of

CENP-C, have not been identified in *D. melanogaster* or *C. elegans* (Oegema et al., 2001; Heeger et al., 2005; Orr and Sunkel, 2011). In addition to these 16, many other proteins have been found to copurify with CENP-A chromatin but their centromere localization is either not constitutive or not yet verified (Obuse et al., 2004; Izuta et al., 2006).

The CCAN complex localizes at the inner kinetochore and creates a bridge between centromeric chromatin and the kinetochore proteins that bind plus ends of spindle microtubule (McAinsh and Meraldi, 2011; Perpelescu and Fukagawa, 2011). While most proteins within the CCAN are interdependent, detailed analysis has assigned specific roles to individual components. The specific roles of the most relevant and best characterized CCAN proteins will be discussed below.

2.3.1. CENP-C and CENP-N bind directly to CENP-A nucleosomes

In vitro experiments have shown that the CENP-C and CENP-N proteins bind to distinct domains of CENP-A to direct the assembly of other CCAN and kinetochore proteins (Carroll et al., 2009, 2010). While CENP-C recognizes the C-terminal LEEGLG motif of CENP-A, CENP-N recognizes the CATD domain embedded in the HFD of CENP-A (Carroll et al., 2009, 2010).

CENP-C is a DNA binding protein that localizes to the inner kinetochore (Saitoh et al., 1992). CENP-C homologues have been identified in virtually all model organisms, including yeast, flies, plants and mammals, and have been shown to be required for proper chromosome segregation and mitotic progression (Tomkiel et al., 1994; Dawe et al., 1999; Fukagawa et al., 2001; Moore and Roth, 2001; Oegema et al., 2001; Ogura et al., 2004; Schuh et al., 2007; Erhardt et al., 2008; Orr and Sunkel, 2011). The centromere localization of CENP-C depends on CENP-A (Oegema et al., 2001; Liu et al., 2006; Hori et al., 2008), and, at least in flies and to a lesser extend in human cells, centromere localization of CENP-A reciprocally requires CENP-C (Erhardt et al., 2008; Carroll

et al., 2010). CENP-C is required for the centromere localization of several kinetochore proteins, including Knl1, the Mis12 complex and the Ndc80 complex that together are known as the KMN network, which forms the principal microtubule binding complex in the kinetochore [(Cheeseman et al., 2006; Milks et al., 2009; Przewloka et al., 2011; Screpanti et al., 2011); see also Section 2.4]. CENP-C is also required for the recruitment of checkpoint proteins, for mitotic checkpoint function (Kwon et al., 2007; Przewloka et al., 2011; Screpanti et al., 2011), and for the centromere localization of other CCAN components such as CENP-H, CENP-I, CENP-K and CENP-T (Carroll et al., 2010).

CENP-N is also required for proper chromosome segregation and mitotic progression (Foltz et al., 2006; McClelland et al., 2007; Carroll et al., 2009). This protein is required for centromere localization of several CCAN components, including CENP-H, CENP-I, CENP-K, CENP-C and CENP-O (McClelland et al., 2007; Carroll et al., 2009). However, depletion of CENP-N did not affect the levels of the Nnf1 component of the Mis12 complex (McClelland et al., 2007). This observation suggests that CENP-N is not directly involved in recruiting kinetochore proteins that bind to spindle microtubules during mitosis. In Chapter 2 of this thesis we will analyze and discuss the additional function of CENP-N in assembling centromeric chromatin.

The fact that both CENP-N and CENP-C bind directly to CENP-A-containing nucleosomes suggests they play a central role in the early stages of centromere/kinetochore assembly (Figure 1.7). Indeed, a recent study showed that CENP-C when artificially tethered onto chromosomes together with CENP-T/W, is sufficient to drive the formation of a functional centromere/kinetochore complex, bypassing the need for CENP-A chromatin (Gascoigne et al., 2011).

2.3.2. CENP-T, CENP-W, CENP-S, and CENP-X form a nucleosome like structure able to warp centromeric DNA

CENP-T was initially identified as a component that copurified with CENP-A chromatin (Obuse et al., 2004; Foltz et al., 2006). Those affinity purifications also identified CENP-M and CENP-U which, when tagged and purified, leaded to the identification of CENP-S (Foltz et al., 2006), indicating that CENP-S is not directly associated with CENP-A nucleosomes. Later, CENP-W and CENP-X were discovered as binding partners of CENP-T and CENP-S, respectively (Hori et al., 2008; Amano et al., 2009). Strikingly, these four CCAN proteins all carry a histone fold domain (HFD) and a recent report showed that, *in vitro*, they form a heterotetrameric complex capable of binding and wrapping centromeric DNA [Figure 1.7; (Nishino et al., 2012)]. The resolution of the crystal structure of this heterotetrameric complex revealed that CENP-T/W and CENP-S/X dimerize through their HFDs. The interface between the CENP-T/W and CENP-S/X heterodimers occurs in the regions of CENP-T and CENP-S that are similar to those involved in heterotetramerization of histones (Nishino et al., 2012). An important difference between these complexes and normal histone complexes is that the histone H3-H4 heterotetramer is a dimer of heterodimers and is therefore symmetric across the tetramerization interface. In contrast, the CENP-T/W/S/X complex is asymmetric. Mutations in the tetramerization interface in either CENP-T or CENP-S abolish their recruitment to centromeres (Nishino et al., 2012), suggesting that these complexes are formed *in vivo* and are important for centromere/kinetochore establishment and function. The discovery that the CENP-T/W/S/X complex is able to bind DNA in a nucleosome-like fashion suggests that functional centromeric chromatin is composed by four different chromatin-binding complexes: H3.1-, H3.3- and CENP-A-containing nucleosomes as well as CENP-T/W/S/X complexes.

Importantly, a portion of CENP-T that is N-terminal to the HFD extends toward the kinetochore to directly bind the Ndc80 complex [Figure 1.7;

(Gascoigne et al., 2011)]. Consistently, CENP-T and CENP-W are loaded and become enriched at centromeres during late S and G2 phases (Prendergast et al., 2011), just before the recruitment of the KMN network to the kinetochores. Another connection of the CCAN to the Ndc80 complex is built through CENP-C, which links the CENP-A nucleosome to the KMN network through its binding to the Mis12 complex (Screpanti et al., 2011). All together, these results indicate that CENP-C and the CENP-T/W/S/X complex form the primary platform that recruits the KMN network (Figure 1.7).

2.3.3. CENP-H/I/K complex

One CCAN subcomplex that localizes to the centromere downstream of the CCAN proteins described above is the CENP-H complex, composed of CENP-H, CENP-I and CENP-K proteins. The centromere localization of this complex has been shown to be dependent on CENP-A, CENP-N, CENP-M, CENP-C, CENP-T and CENP-L (Foltz et al., 2006; Okada et al., 2006; McClelland et al., 2007; Hori et al., 2008). The CENP-H/I/K complex is required for the centromere localization of the CENP-O/P/Q/R/U complex and of the KNM network (Liu et al., 2003; Okada et al., 2006; Kwon et al., 2007; Cheeseman et al., 2008; Hori et al., 2008). Accordingly, depletion of any component of the CENP-H/I/K complex induces severe defects in kinetochore assembly and chromosome segregation (Nishihashi et al., 2002; Okada et al., 2006).

Besides their role in recruiting other centromere/kinetochore proteins, CENP-H and CENP-I interact with the plus end of kinetochore microtubules modulating their turnover rate and promoting accurate chromosome alignment at the metaphase plate (Amaro et al., 2010). The CENP-H/I/K complex also has a role in recruiting CENP-A to the centromere as will be described in the Chapter 2 of this thesis.

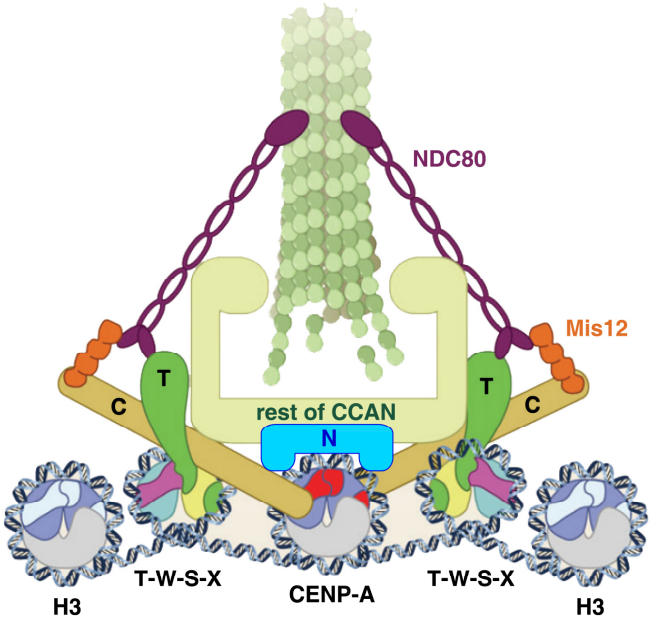

Figure 1.7. The CCAN forms a bridge between centromeric DNA and microtubule plus ends. The CENP-T/W/S/X complex links centromeric chromatin to the mitotic kinetochore through a direct interaction between CENP-T and the NDC80 microtubule-binding complex. The CENP-A nucleosome is contacted by CENP-C and CENP-N that bind to the Mis12 complex and the remaining CCAN, respectively. In this way, parallel contacts between centromeric DNA and microtubules are established. Adapted from Foltz and Stukenberg, 2012.

2.4. The Kinetochore

The kinetochore is a highly complex proteinacious structure involved in several mitotic functions, most importantly in attachment to spindle microtubules and in mitotic checkpoint signaling (Rieder and Salmon, 1998). Despite a few minor organism-specific differences, kinetochore composition and organization appears to be highly conserved among most eukaryotes (Musacchio and Salmon, 2007; Gascoigne and Cheeseman, 2011). Kinetochore proteins are targeted to the centromere during G2, prophase or other specific stages of

mitosis, and are disassembled following microtubule attachment, after anaphase onset or when mitosis is completed (Cheeseman and Desai, 2008).

One key outer kinetochore component critical for the formation of functional kinetochores is the KMN network, which acts as a connector between the CCAN and the mitotic spindle by providing a direct interaction to spindle microtubules [Figures 1.7 and 1.8 B; (Cheeseman et al., 2006; Cheeseman and Desai, 2008; Tanaka and Desai, 2008)]. As mentioned above, the KMN network is composed of three distinct proteins or complexes: the Knl1 protein (or Blinkin), the Mis12 complex (Mis12, Nnf1, Nsl1, and Dsn1) and the Ndc80 complex (Spc24, Spc25, Nuf2, and Ndc80) (Ciferri et al., 2005; Wei et al., 2005; Cheeseman et al., 2006; Kline et al., 2006; Kiyomitsu et al., 2007).

In addition to directing kinetochore-microtubule attachment, the KMN network also forms the basis for the binding of a number of other proteins that transiently localize at the outer kinetochore during prometaphase and are involved in mitotic checkpoint signaling and microtubule stability (DeLuca et al., 2003; Kline et al., 2006; Liu et al., 2006; Kiyomitsu et al., 2007). Among these are two important motor proteins: the plus end-directed microtubule motor CENP-E and the minus end-directed microtubule motor Dynein (Mao et al., 2010). These motor proteins play important roles in kinetochore-microtubule attachment, chromosome congression and mitotic checkpoint control (Przewloka and Glover, 2009; Mao et al., 2010). CENP-E and Dynein are recruited to the kinetochore by other outer kinetochore proteins, which include CENP-F, in the case of CENP-E, and Spindly and the RZZ complex (ROD, ZWILCH, and ZW10), in the case of Dynein (Mao et al., 2010). Another set of proteins that associate with the kinetochore during prometaphase, is a group of microtubule-associated proteins (MAPs) that bind the plus end of microtubules, promoting their stabilization (Przewloka and Glover, 2009). In addition, mitotic checkpoint components localize at unattached kinetochores during mitosis to generate the mitotic checkpoint signal (Figure 1.8 A), as discussed further below (Musacchio and Salmon, 2007).

2.4.1. Mitotic checkpoint and metaphase to anaphase transition

The mitotic checkpoint is a molecular surveillance system that ensures accurate segregation of mitotic chromosomes by delaying anaphase onset until all chromosomes are stably attached to the mitotic spindle, via kinetochore-microtubule interactions, in a bipolar manner (Gorbsky, 2001; Musacchio and Salmon, 2007). The mitotic checkpoint is active in the presence of unattached or improperly attached chromosomes and thereby monitors the status of microtubule attachment (Figure 1.8 A). Strikingly, a single unattached kinetochore is sufficient to maintain mitotic checkpoint signaling (Rieder et al., 1995). When active, the mitotic checkpoint produces a 'wait-anaphase' signal capable of delaying anaphase onset (Yu, 2002; Cleveland et al., 2003; Rieder and Maiato, 2004). This signal is initiated and executed by several mitotic checkpoint proteins: Mad1, Mad2, Bub1, Bub3, BubR1 and Mps1 (Figure 1.8 A). These proteins are specifically targeted to kinetochores that lack tension and/or microtubule attachment (Musacchio and Salmon, 2007; Kops, 2008).

The mitotic checkpoint prevents anaphase by inhibiting the anaphase-promoting complex/cyclosome (APC/C) (Yu, 2002). This complex is an ubiquitin ligase that promotes the metaphase to anaphase transition by driving proteasome-mediated degradation of mitotic proteins that include cyclin B and securin, leading to downregulation of Cdk1 and to sister chromatid separation, respectively (reviewed in Peters, 2006). The activity of the APC/C is controlled primarily by two activator subunits: Cdc20, at the metaphase to anaphase transition, and Cdh1 during late mitosis and G1 phase (Peters, 2006). When the mitotic checkpoint is active, the mitotic checkpoint complex (MCC), which is composed of BubRI, Mad2 and Bub3, and whose production is catalyzed by unattached kinetochores, binds to the APC/C activator Cdc20 [Figure 1.8 A; (Musacchio and Salmon, 2007; Kulukian et al., 2009)]. This interaction of the MCC with Cdc20 induces a conformational change in the APC/C complex that prevents the binding and ubiquitination of its substrates (Herzog et al., 2009).

When all chromosomes are properly attached in a bipolar manner to the mitotic spindle, the mitotic checkpoint is silenced through action of the p31^{comet} protein and removal of Mad1 and Mad2 from the kinetochores, which is dependent on the motor protein Dynein (Figure 1.8 B). This will lead to activation of the APC/C^{Cdc20} and to anaphase onset (Hagan et al., 2011; Kim and Yu, 2011).

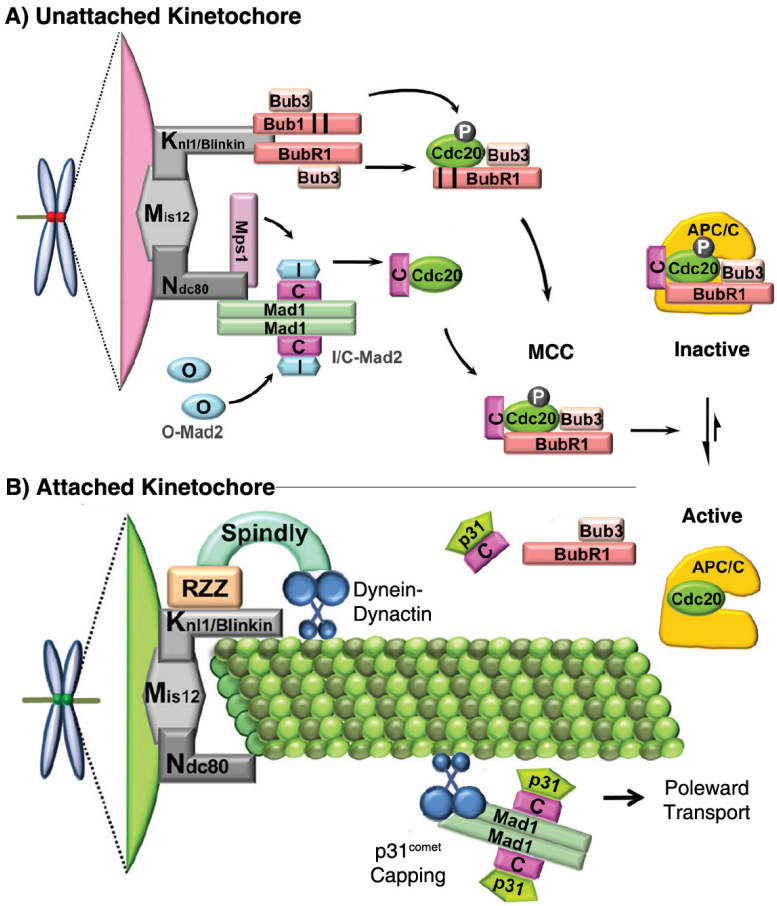

Figure 1.8. APC/C inhibitory signals generated by the mitotic checkpoint. A) The mitotic checkpoint signal is maintained by the presence of unattached kinetochores. When the mitotic checkpoint is active Mad2, and BubR1 bind to Cdc20, forming a larger mitotic checkpoint complex (MCC; BubR1-Bub3-Mad2-Cdc20) that inhibits APC/C^{Cdc20}. Unattached kinetochores recruit and activate Mad2 and BubR1 to promote MCC assembly. Continued on the next page.

Figure 1.8. (continued) The KMN network (Knl1-Mis12-Ndc80) forms a platform for the recruitment of checkpoint proteins like Bub1 and BubR1. The kinetochore targeting of the Mad1-Mad2 core complex depends on Mps1 and Ndc80. Kinetochore-bound Mad1-Mad2 catalyzes conformational activation of Mad2 [open (O) to close (C), I is intermediate]. The MCC binds to and inhibits the APC/C^{Cdc20} by blocking substrate recognition. Bub1 phosphorylates Cdc20 and also contributes to APC/C^{Cdc20} inhibition. B) Upon microtubule attachment the mitotic checkpoint is inactivated. This inactivation requires $p31^{comet}$ binding to the active form of Mad2 (C) in the cytosol as well as to Mad2 in the Mad1-Mad2 core complex thereby blocking the recruitment and activation of cytosolic inactive Mad2 (O). Checkpoint silencing is also facilitated by removal of Mad1-Mad2 from kinetochores, which is performed by the microtubule motor Dynein. Dynein-Dynactin is recruited to properly attached kinetochores by RZZ and Spindly, and mediates the poleward transport of Mad1-Mad2. Adapted from Kim and Yu, 2011.

3. Epigenetic inheritance of centromeres

3.1. The basic concept of Epigenetics

Historically, the word “epigenetics” was used to describe events that could not be explained directly by genetic principles. Conrad Waddington, who coined the term, defined epigenetics as “the branch of biology which studies the causal interactions between genes and their products, which bring the phenotype into being” (Waddington, 1942). More recently, the term epigenetics has been used to define “the study of mitotically and/or meiotically heritable changes in gene function that cannot be explained by changes in DNA sequence” (Riggs et al., 1996).

Over the years, numerous biological phenomena, some considered bizarre and inexplicable, have been lumped into the category of epigenetics. Examples of these epigenetic phenomena are paramutation in maize (an interaction between two alleles in which one allele causes heritable changes in the other allele), position effect variegation in the fruit fly *Drosophila melanogaster* (in which the local chromatin environment of a gene determines its expression), and the imprinting of specific paternal or maternal loci in mammals (Allis et al., 2007).

During the last decade, there has been a significant advance in our understanding of the molecular mechanisms underlying epigenetic phenomena (Bird, 2007). Many studies have shown that epigenetic processes involve DNA methylation, histone modifications, histone variants, nucleosome remodeling and higher order chromatin reorganization (Bird, 2002; Imhof, 2006; Kouzarides, 2007). These epigenetic modifications collectively maintain a unique profile of gene expression in each cell lineage and thereby maintain cellular identity. Epigenetic modifications within a chromosome are important, not only for regulation of gene expression, but also for maintaining the identity of important chromosomal structures, like telomeres and centromeres (Allis et al., 2007). In order to maintain the identity of these chromosomal structures and to maintain

cellular identity, epigenetic information must be replicated throughout cell divisions (Probst et al., 2009; Blomen and Boonstra, 2011). Indeed, although most epigenetic modifications are reversible, they can be maintained by stable transmission or by continued, self-directed recruitment of modifying protein complexes that thereby maintain epigenetic marks throughout the cell cycle.

3.1.1. Inheritance of DNA methylation during DNA replication

DNA methylation is perhaps the best characterized chemical modification of chromatin and is a stable, heritable and critical component of epigenetic regulation. In mammals, nearly all DNA methylation occurs on cytosine residues of CpG dinucleotides. Regions of the genome that have a high density of CpGs are referred to as CpG islands, and DNA methylation of these islands correlates with transcriptional repression (Goll and Bestor, 2005). DNA methylation plays a critical role in gene regulation and chromatin organization during embryogenesis and gametogenesis, typically through the establishment of long-term repression (Goll and Bestor, 2005; Surani et al., 2007). In addition, DNA methylation plays a role in heterochromatin formation, X chromosome inactivation, allelic exclusion, and parental gene imprinting (Yang and Kuroda, 2007).

DNA and its methylation marks are replicated using semiconservative mechanisms of inheritance, in which local information is copied from a template (Probst et al., 2009). During S phase, replication of the two parental DNA strands, results in two hemimethylated strands and two newly synthesized strands. The maintenance of DNA methylation throughout the cell cycle is mainly mediated by three DNA methyltransferases (DNMTs): DNMT1, DNMT3a and DNMT3b (Jones and Liang, 2009). DNMT1 is considered the primary DNMT involved in the inheritance of DNA methylation due to its affinity towards hemimethylated DNA (Hermann et al., 2004). During DNA replication, DNMT1 associates with the proliferating cell nuclear antigen (PCNA), a component of the replication machinery (Moldovan et al., 2007), and with NP95, a protein that specifically

binds hemimethylated DNA, and subsequently restores methylation on the daughter strands (Sharif et al., 2007). Additionally, two *de novo* DNA methyltransferases, DNMT3a and DNMT3b, appear also to be also required for epigenetic inheritance of DNA methylation patterns (Liang et al., 2002; Chen et al., 2003). These DNMTs may be involved in a proofreading mechanism by methylating CpG sites missed by DNMT1 (Riggs and Xiong, 2004; Jones and Liang, 2009).

3.1.2. Histone modifications and inheritance of chromatin states

Histone modifications are considered another important component of the epigenetic machinery. Histones can be modified by acetylation, methylation, phosphorylation, ubiquitination, sumoylation, ADP-ribosylation, deimination, and proline isomerization (Kouzarides, 2007). These modifications can directly alter histone or chromatin structure, thereby restricting or facilitating the access to transcription factors or transcription machinery. Moreover, these modifications can recruit other proteins or protein complexes, for example the heterochromatin-associated protein 1 (HP1), Polycomb repressive complexes (PcG) or the Trithorax group proteins (TrxG), to induce a repressive or accessible chromatin state (Strahl and Allis, 2000).

During the last decade, various studies have uncovered a correlation between certain histone modifications and transcriptional states. However, only a subset of these histone modifications, most notably histone methylation, appears to be associated with epigenetic inheritance of certain transcriptional states. Nevertheless, it is still unclear whether histone methylation itself is heritable. Generally, histone hyperacetylation and methylation of histone H3 on Lysine 4, 36 and 79 (H3K4, H3K36 and H3K79) are characteristic of transcriptionally active euchromatin. On the other hand, histone hypoacetylation, methylation of histone H3 on Lysine 9, 27 and 64 (H3K9, H3K27 and H3K64), and methylation of histone H4 on Lysine 20 (H4K20) are associated with transcriptionally repressive

heterochromatin. Besides their role in transcription regulation, histone modifications are also involved in DNA repair, DNA replication and chromosome condensation (Kouzarides, 2007).

In order to be heritable and therefore to qualify as epigenetic marks, the histones and their modifications must be accurately replicated during DNA replication (Groth et al., 2007). Thus, in order to avoid loss of information that is encoded in histone modifications, it is crucial to have a tight coordination between the recycling of parental histones H3 and H4 (along with their histone marks) and the assembly of newly synthesized unmodified histones (Groth et al., 2007). If the modifications of new histones are guided by modifications of parental histones, the latter ones must be distributed between the two sister chromatids during DNA replication (Probst et al., 2009). Maintenance of histone modifications could be achieved by using a neighboring histone as a template. This would likely involve recognition of the parental modification by a chromatin-binding protein ("reader" protein), that in turn modifies or recruits a chromatin modifier ("writer" protein) that will copy the histone modification to the newly incorporated histone (Taverna et al., 2007).

Indeed, this "reader-writer" mechanism has been suggested as a self-reinforcing loop in the maintenance of HP1 at pericentromeric heterochromatin (Bannister et al., 2001; Lachner et al., 2001). HP1 is required for maintenance of heterochromatin and it binds ("reads"), through its chromodomain, to di- and trimethylated H3K9 residues (Fischle et al., 2003), an epigenetic mark that is enriched in heterochromatin (Grewal and Jia, 2007). HP1 associates with SUV39H1, the major Lysine methyltransferase (KMT) responsible for trimethymation of H3K9 (Aagaard et al., 1999; Rea et al., 2000). This KMT acts as a "writer" for this particular histone modification.

3.1.2. Polycomb and Trithorax family of proteins as key players in epigenetic control of gene expression

Epigenetic regulation of gene expression also involves the Polycomb and Trithorax (PcG/TrxG) family of proteins that were initially identified in *D. melanogaster* (Schwartz and Pirrotta, 2007). PcG and TrxG are recruited to chromatin by DNA regulatory elements called PcG or TrxG regulatory elements (PREs or TREs, respectively) to mediate epigenetic inheritance of silent and active chromatin states, respectively (Schwartz and Pirrotta, 2007). PcG and TrxG proteins are implicated in stem cell identity, cancer, genomic imprinting in plants and mammals, and X chromosome inactivation (Schuettengruber et al., 2007).

PcG and TrxG proteins function in distinct multiprotein complexes and act as chromatin modifiers, mainly, by inducing covalent modifications of histones. The Polycomb system mediates silencing through two distinct complexes: Polycomb repressive complex 1 (PRC1) and PRC2. Although these complexes share some functional redundancy (Leeb et al., 2010), their mechanisms of action are rather distinct. PRC2 contains the histone Lysine methyltransferase EZH2, which trimethylates Lysine 27 of histone H3 (Cao et al., 2002; Kuzmichev et al., 2002). Similar to the maintenance of HP1 and H3K9me3, the repressive methylation mark H3K27me3 is thought to be maintained during DNA replication by a self-propagating loop, in which PRC2 binds to the H3K27me3 mark and replicates it by modifying neighboring nucleosomes (Hansen et al., 2008).

H3K27me3 also serves as a recruitment site for PRC1 (Wang et al., 2004a), which binds to this histone mark via the chromodomain of one of its members, the Polycomb protein (PC) (Cao and Zhang, 2004). PRC1 is responsible for the deposition of a second histone mark, via the E3 ubiquitin ligase activity of RING1 and RING2 in mammals, which mono-ubiquitinates Lysine 119 of histone H2A (H2AK119ub). The H2AK119ub chromatin mark can inhibit transcription through inhibition of RNA polymerase II elongation (Stock et

al., 2007; Zhou et al., 2008). How PRC1 is inherited across cell divisions is not well understood although it has been shown remain bound to chromatin even during DNA replication *in vitro* (Francis et al., 2009).

Unlike the PcG proteins that mediate the epigenetic repression of genes, the TrxG proteins function in an antagonistic fashion and are implicated in transcriptional activation (Blomen and Boonstra, 2011). Interestingly, several members of the TrxG complex are histone Lysine methyltransferases that trimethylate Lysine 4 of histone H3 (H3K4me3) (Byrd and Shearn, 2003; Gregory et al., 2007; Schwartz and Pirrotta, 2007), which is a mark of transcriptionally active chromatin. In addition, TrxG is implicated in H3K27 acetylation that is also implicated in gene activation and competes with the repressive H3K27me3 mark (Tie et al., 2009; Schwartz et al., 2010). TrxG has also been proposed to play a global role in transcriptional elongation (Smith et al., 2004; Petruk et al., 2006).

It is well established that PcGs and TrxG contain modifying enzymes that create histone modifications, which are associated with inactive or active genes, respectively. However, it is unclear how these repressive or active gene states are stably inherited throughout cell divisions. Current evidence does not discriminate between models where inheritance is mediated by the histone modifications from those in which the effector PcG and TrxG complexes are themselves inherited.

3.1.2. Other players in epigenetic processes

Another important class of epigenetic machinery are noncoding RNAs (Bernstein and Allis, 2005). Clear examples of RNA involvement range from dosage compensation mechanisms, in *D. melanogaster* and mammals, mediated by the *rox* and *XIST* RNAs, respectively, to the silencing of both genes and repetitive DNA sequences by RNAi-related pathways. These RNAs often act together with chromatin modifications and DNA methylation machinery to achieve stable silencing (Bernstein and Allis, 2005). Noncoding RNAs appear powerful

factors in the maintenance of epigenetic states because, as soluble molecules, they can be inherited into subsequence cell generations efficiently. In addition, they have an intrinsic capacity to confer specificity through sequence complementarity (Moazed, 2011).

Chromatin remodeling and the incorporation of specialized histone variants are also implicated in epigenetic regulation and inheritance. ATP-dependent chromatin remodeling complexes are thought to modify chromatin accessibility by altering histone-DNA interactions, perhaps by sliding or ejecting nucleosomes (Smith and Peterson, 2005). Histone variants, including H3.3, CENP-A and H2A.Z are associated with specific chromosomal domains, and this association depends on dedicated chaperones and exchange factors (Henikoff and Ahmad, 2005; Polo and Almouzni, 2006). The histones H3.3 and H2A.Z have been implicated in transcription activation, while CENP-A, discussed further below, has been shown to have a crucial role in maintaining centromere identity (Henikoff and Ahmad, 2005; Ekwall, 2007).

3.2. Centromere as a show case to study epigenetic inheritance

Centromeres in the budding yeast *S. cerevisiae* are specified by a specific 125 bp sequence that is sufficient for centromere formation. In higher eukaryotes, centromeres are normally associated with long stretches of repetitive AT-rich sequences as described in Section 2.1. Based on the early yeast model it was initially proposed that these DNA sequences associated with centromeric chromatin were required for centromere function and identity (Masumoto et al., 1989; Grady et al., 1992; Tyler-Smith et al., 1993). This view appeared further supported by the finding that one of the constitutive centromere proteins, the centromere protein B (CENP-B), specifically binds to centromeric alphoid repeats (Masumoto et al., 1989; Muro et al., 1992). However, subsequent work showed that CENP-B is a nonessential protein in mice (Hudson et al., 1998; Kapoor et al., 1998; Baum and Clarke, 2000). Moreover, the highly repetitive tandem sequence

repeats at large regional centromeres, in fission yeast and metazoans, are not conserved among species, or even among different chromosomes of an individual organism (Karpen and Allshire, 1997; Choo, 2001). These observations led to the proposal that centromere function and propagation may not be dependent on any specific DNA sequence and, instead, is controlled by an epigenetic mechanism (Vafa and Sullivan, 1997; Warburton et al., 1997).

Evidence in support of an epigenetically maintained centromere is the stably transmission of dicentric chromosomes in humans and flies (Sullivan and Willard, 1998; Higgins et al., 1999). Dicentric chromosomes contain two regions of centromeric DNA, which are capable of functioning as active centromeres. If both centromeres are functional this will result in anaphase chromosome bridging, chromosome breakage and chromosome loss, as is observed in dicentric chromosomes in maize (McClintock, 1939). In cases of stable inheritance of dicentric chromosomes this fate is avoided by the functional inactivation of one centromere (Sullivan and Schwartz, 1995; Faulkner et al., 1998; Sullivan and Willard, 1998; Agudo et al., 2000). This shows that the presence of centromeric DNA on a chromosome is not sufficient for centromere function.

More direct evidence demonstrating the epigenetic nature of the centromere was provided by the discovery of a newly formed, ectopic human centromeres (Voullaire et al., 1993). In contrast to natural centromeres, which are formed within tandemly repeated α-satellite DNA, this new centromere (or neocentromere) lacked any characteristic centromeric DNA sequences and had formed in a gene-rich area of the genome. Therefore, centromeric DNA is not required per se for the formation and maintenance of a functional centromere. Since the identification of the first neocentromere, almost 100 cases have been identified (Warburton, 2004; Marshall et al., 2008). Human neocentromeres occur naturally and are typically identified through prenatal screening or by cytogenetic analyses of individuals with a developmental delay or congenital abnormalities (Depinet et al., 1997). All known essential centromere/kinetochore proteins are

present at neocentromeres and these proteins behave largely identical to their α-satellite-DNA-based counterparts in mitosis and meiosis (Saffery et al., 2000; Marshall et al., 2008; Bassett et al., 2010). However, the size of the CENP-A domain at neocentromeres is reduced and ranges from 100 kb to 464 kb, emphasizing the plasticity of CENP-A chromatin (Sullivan, 2001). Furthermore, mapping of neocentromeres does not reveal any particular “hotspots” for neocentromere formation (Alonso et al., 2003; Chueh et al., 2005). Importantly, neocentromeres are mitotically stable and in some cases have been shown to be germ line transmitted (Depinet et al., 1997; du Sart et al., 1997; Warburton et al., 2000). Neocentromere formation has been observed in several other organisms including *D. melanogaster* (Williams et al., 1998; Maggert and Karpen, 2001), *S. pombe* (Ishii et al., 2008), *Triticum aestivum* [wheat; (Nasuda et al., 2005)], *Candida albicans* (Ketel et al., 2009) and *C. elegans* (Yuen et al., 2011). All together these results indicate that, in most eukaryotes, centromeres are epigenetically defined and inherited.

3.2.1. CENP-A as a key epigenetic mark

As discussed in Section 2.2.1, centromeric chromatin is characterized by the presence of a specific histone H3 variant called CENP-A. The evolutionary conservation of CENP-A among all eukaryotes and the constitutive centromeric localization of this histone throughout the cell cycle on all active centromeres, including neocentromeres, make CENP-A a strong candidate for a primary role in specification and propagation of the site of kinetochore assembly (Allshire and Karpen, 2008; Silva and Jansen, 2009). Supporting this idea, CENP-A nucleosomes are extremely stable, being turned over only by dilution and redistribution between sister chromatids during DNA replication (Jansen et al., 2007; Hemmerich et al., 2008; Dunleavy et al., 2011). Moreover, CENP-A is required for establishment and function of kinetochores in various organisms. Depleting CENP-A protein in yeasts, worms, flies and mammals severely disrupts

mitosis and cell cycle progression (Meluh et al., 1998; Howman et al., 2000; Blower and Karpen, 2001; Oegema et al., 2001). Additionally, as discussed in section 2.2.1, inactivating or deleting CENP-A causes mislocalization of most centromere/kinetochore proteins, which indicates that CENP-A is a central component in centromere/kinetochore assembly. On the other hand, overexpression of CENP-A^{CID} in *D. melanogaster* leads to its misincorporation into noncentromeric chromatin, which in turn triggers the formation of ectopic, functional centromeres/kinetochores (Heun et al., 2006). Once formed, these new centromeres can recruit newly synthesized CENP-A^{CID}, indicating that this centromere mark can be temporarily inherited in an epigenetic fashion after seeding the ectopic centromere (Olszak et al., 2011). Additionally, it was shown that these ectopic centromeres form preferentially in the vicinity, but not overlapping with heterochromatin regions, such as telomeres and pericentromeric heterochromatin (Olszak et al., 2011). However, why silent domains proximal to heterochromatin are particularly suited for *de novo* formation of kinetochores remains unclear. One possibility is that this silent chromatin state mimics the chromatin environment of an endogenous centromere.

More recently, simultaneous studies in fly and human cells provided direct evidence that CENP-A functions as a seed, which is required and sufficient to drive the formation of functional centromeres/kinetochores (Barnhart et al., 2011; Mendiburo et al., 2011). Both studies employed the LacI-LacO tethering system, which consists of the integration of bacterial Lac operator (LacO) sequences into a defined chromosomal locus to form a binding platform for the ectopically expressed Lac repressor protein (LacI). Tethering of a CENP-A^{CID}-GFP-LacI fusion protein to stably integrated LacO arrays, in *Drosophila* S2 cells, led to the formation of ectopic and functional centromeres/kinetochores (Mendiburo et al., 2011). These ectopic, LacO associated centromeres direct the incorporation of CENP-A^{CID} that lack the LacI-anchor (Figure 1.9), providing direct evidence that this epigenetic mark is able to self-propagate in order to maintain centromere identity. In human cells, a similar approach was used to tether a LacI fusion of

HJURP, the CENP-A specific chaperone, to a LacO array at a noncentromeric locus. This resulted in the stable recruitment of CENP-A to LacO arrays (Figure 1.9), which was sufficient to nucleate active centromeres/kinetochores that capture spindle microtubules and allow chromosome movement, as was shown for recruitment of CENP-A^{CID} itself (Barnhart et al., 2011).

In these two tethering experiments, the ectopic centromeres were created in addition to the original centromere, leading to mitotic failure and cell death. Therefore, although these approaches show that artificial nucleation of CENP-A or HJURP results in formation of functional centromeres and kinetochores, they fail to show whether these ectopic centromeres are heritable across cell generations. Mendiburo *et al.* addressed this using plasmid-based-artificial chromosomes that contain LacO arrays in *Drosophila* S2 cells (Mendiburo et al., 2011). These plasmids can be replicated but not segregated, leading to rapid loss from a dividing population of cells. Tethering of CENP-A^{CID}-GFP-LacI to these plasmids led to recruitment of centromere/kinetochore proteins and microtubule binding. This resulted in stable transmission of the plasmids for several cell divisions even after eliminating the CENP-A^{CID}-GFP-LacI seed that initiated the centromere (Mendiburo et al., 2011).

Combined, these results demonstrate that CENP-A has the capacity to initiate an epigenetic feedback mechanism responsible for propagation and stable inheritance of centromere identity. If CENP-A is the seed that sows the epigenetic centromere, HJURP is the seed-carrier that allows self-propagation of this epigenetic mark by promoting assembly of new CENP-A at sites where old copies are already assembled. Determining how CENP-A is replicated and maintained becomes crucial to understand how centromeres are epigenetically inherited across cell divisions.

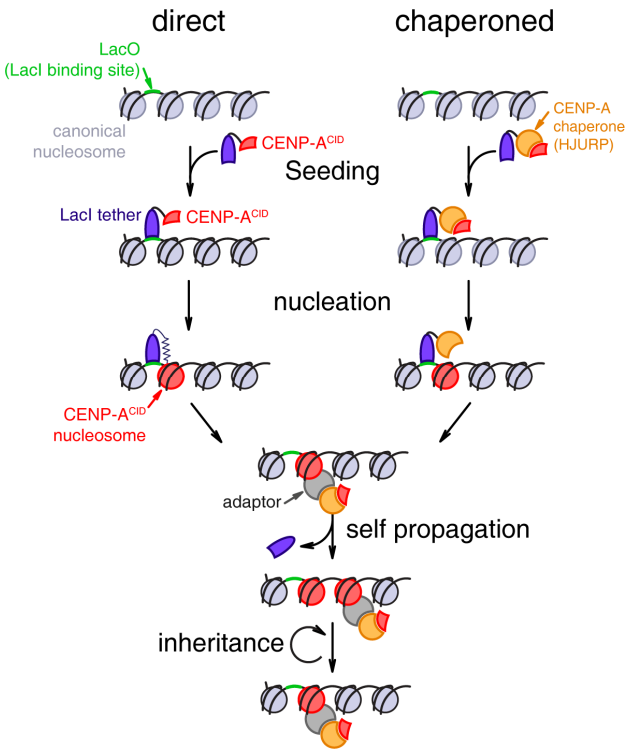

Figure 1.9. Seeding and propagation of centromeric chromatin. CENP-A is targeted to a naive chromatin locus either by direct fusion to locally bound LacI (direct; Mendiburo et al., 2011) or through recruitment by the LacI-tethered chaperone HJURP (chaperoned; Barnhart et al., 2011). This results in the nucleation of CENP-A nucleosomes, which in turn triggers the propagation of CENP-A chromatin in a self-templating manner (likely through an adaptor intermediate) without the need for the initial LacI-seed. CENP-A chromatin propagation and turnover through cell divisions reach an equilibrium resulting in stable inheritance of the epigenetic centromere mark.

3.2.2. CENP-A maintenance and assembly during the cell cycle

The assembly of canonical chromatin occurs during S phase and requires tight coupling between DNA replication and histone H3.1 synthesis (Wu and Bonner, 1981). It was initially proposed that centromeric DNA replication and CENP-A synthesis are also coupled but occur temporally separated from general chromatin assembly, during late S phase, to avoid competition between the two

distinct assembly mechanisms (Shelby et al., 1997; Csink and Henikoff, 1998). However, this hypothesis was later invalidated by the findings that in human cells CENP-A is synthesized in G2 phase and can be assembled independently of DNA replication (Shelby et al., 2000). Thus, unlike assembly of H3.1 in general chromatin, CENP-A assembly into centromeric chromatin occurs outside S phase. During DNA replication, both H3.1 and CENP-A nucleosomes are distributed between the two sister chromatids (Shelby et al., 2000; Annunziato, 2005; Jansen et al., 2007; Xu et al., 2010), ensuring that the epigenetic information carried in these nucleosomes is propagated across cell divisions. It was then proposed that a CENP-A-specific assembly factor recognizes and propagates CENP-A containing chromatin during G2 phase (Shelby et al., 2000; Sullivan, 2001). Although CENP-A is crucial for centromere function and propagation, misincorporation of CENP-A and CENP-C at ectopic noncentromeric sites, caused by overexpression of CENP-A in human cells, was not sufficient to form functional and heritable centromeres at these ectopic sites (Van Hooser et al., 2001). These results led to the proposal that additional mechanisms such as centromere function itself are required to mark active centromeres for propagation (Malik and Henikoff, 2002; Mellone and Allshire, 2003). These authors suggested that either kinetochore-microtubule attachment or the tension generated between sister chromatids before anaphase onset marks the centromere location in a way that cannot be simply reproduced by ectopic recruitment of CENP-A or other centromeric proteins (Malik and Henikoff, 2002; Mellone and Allshire, 2003). This hypothesis predicts CENP-A assembly to occur at or after metaphase.

Since CENP-A is present at centromeres throughout the cell cycle determining the timing of its assembly requires specific tools to assess protein dynamics. One powerful tool is the fluorescent pulse labeling approach based on SNAP-tagging in human cells (Keppler et al., 2003, 2004). This labeling technique allows for specific visualization of a newly synthesized pool of CENP-A and will be introduced in more detail in Chapter 2. These experiments showed

that SNAP tagged CENP-A, which is expressed throughout the cell cycle, is targeted to centromeres only during late telophase/early G1 phase of the cell cycle (Figure 1.10; Jansen et al., 2007). A similar conclusion was reached by analyzing steady state CENP-A^{CID} levels and by FRAP (fluorescence recovery after photobleaching) in cycling *D. melanogaster* embryos and in human cells (Schuh et al., 2007; Hemmerich et al., 2008). The use of SNAP-tagging to analyze the dynamics of CENP-A also provided direct evidence that CENP-A nucleosomes assembled at centromeres are extremely stable and are semiconservatively distributed to both sister chromatids during DNA replication [Figure 1.10; (Jansen et al., 2007; Dunleavy et al., 2011)].

The finding that CENP-A assembly occurs immediately after the centromere completes its main functions raised the possibility that those functions, which include kinetochore formation, microtubule binding and chromosome segregation, act as signal to initiate centromere propagation as it was previously proposed by Mellone and Allshire (Mellone and Allshire, 2003). However, cells forced to exit mitosis without spindle microtubules were able to load CENP-A at centromeres, indicating that neither microtubule attachment nor tension are required for centromere propagation (Jansen et al., 2007; Schuh et al., 2007). Although this result refutes the Mellone and Allshire hypothesis and reveals that kinetochore function is not essential for centromere propagation, it does not exclude the involvement of the kinetochore in secondary aspects of CENP-A assembly. Indeed, a “kinetochore maintenance mechanism” was recently proposed to regulate the amount of CENP-A that is loaded at each centromere (Brown and Xu, 2009). The untested hypothesis is that separation or stretching of the sister chromatids during metaphase negatively regulates the amount of CENP-A which will be assembled upon mitotic exit. Centromeres that bind few microtubules and therefore display weak tension between sister chromatids will assemble more CENP-A than centromeres that bind many microtubules and exert strong tension. This reciprocal relationship forms the

basis for a negative feedback loop that controls the precise amount of CENP-A assembled during each cell division (Brown and Xu, 2009).

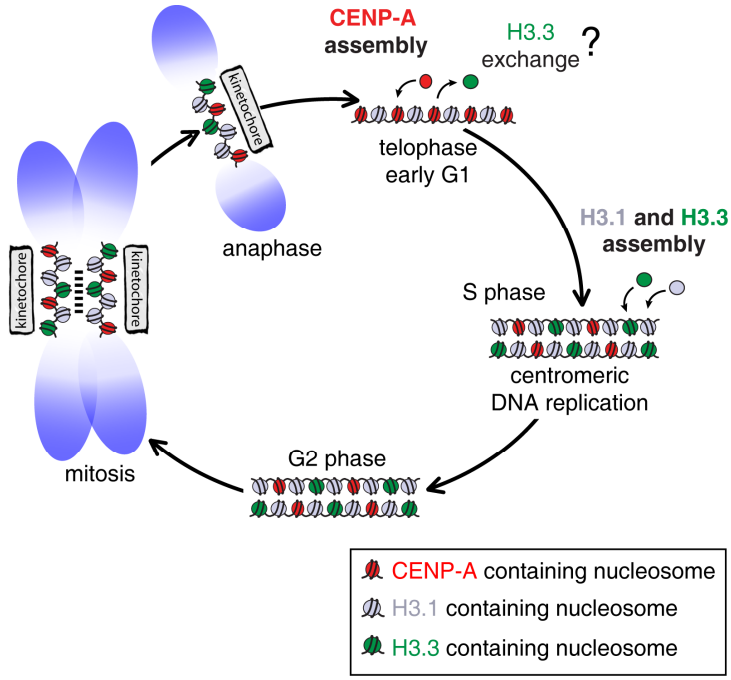

Figure 10. Schematic depicting centromeric chromatin composition across the cell cycle. CENP-A-containing nucleosomes (red) are interspersed with canonical H3.1 containing nucleosomes (light blue). During DNA replication, in S phase, canonical H.3.1 containing nucleosomes are assembled throughout chromosome arms and also at the centromere. During this phase it is thought that H3.3 containing nucleosomes are assembled as "placeholders" of CENP-A nucleosomes (Dunleavy et al., 2011). This mixed set of nucleosomes is the substrate for nucleating kinetochore assembly in mitosis and is maintained until cells exit mitosis. CENP-A assembly initiates in late telophase and proceeds throughout G1 phase (Lagana et al., 2010). This assembly step presumably concurs with excision of H3.3 nucleosomes). CENP-A-, H3.1 and H3.3- containing nucleosomes are stylized as single nucleosomes but may represent continuous alternating arrays of one or the other type. In mitosis, CENP-A nucleosomes may coalesce to form a rigid interface for kinetochore formation as proposed previously (Zinkowski et al., 1991; Blower et al., 2002; Black et al., 2004, 2007a, 2007b). Adapted from Jansen et al., 2007.

Importantly, timing of CENP-A assembly appears to be evolutionary conserved in many eukaryotes. Exit from mitosis is also required for CENP-A loading in *Drosophila* syncytial embryos and in *Xenopus* egg extracts (Schuh et al., 2007; Bernad et al., 2011; Moree et al., 2011). However, in *Drosophila* S2 cells CENP-A assembly occurs during metaphase, indicating that in this specific cell type mitotic exit is not required. I will discuss the possible causes for this difference in Chapter 5. In fission yeast, CENP-A^{Cnp1} is incorporated into centromeric chromatin during two discrete periods of the cell cycle: S and G2 phases (Takahashi et al., 2005; Takayama et al., 2008). However, shortening G2 phase and thus reducing CENP-A^{Cnp1} assembly during this phase has no effect on centromere function, suggesting that G2 deposition is a secondary pathway used perhaps to increase the fidelity of centromere propagation in this organism (Takayama et al., 2008). As a result, the primary wave of centromere propagation appears to occur during S phase in fission yeast. This organism has a very short G1 phase, and consequently S phase occurs immediately following mitotic exit and before cytokinesis (Forsburg and Nurse, 1991). This suggests that cell cycle control of CENP-A^{Cnp1} assembly into fission yeast centromeres occurs through a mechanism that is conserved in most eukaryotes. It is important to note that not only the time of assembly is conserved but also the proteins required to incorporated CENP-A at the centromeres are conserved among different organisms, including fission yeast, *Xenopus*, *C. elegans* and humans (Hayashi et al., 2004; Fujita et al., 2007; Maddox et al., 2007; Dunleavy et al., 2009; Foltz et al., 2009; Pidoux et al., 2009; Williams et al., 2009; Bernad et al., 2011). The identity and the function of these proteins will be discussed in Chapter 2.

4. Cell cycle control

Successful progression through the cell division cycle is characterized by the ordered sequence of events such as DNA replication, spindle assembly, chromosome segregation and cytokinesis (Figure 1.2). To ensure that all cell cycle events occur accurately and in the correct order, eukaryotic cells have developed sophisticated cell cycle control mechanisms. This cell cycle regulation is achieved through a combination of phosphorylation events by cyclin-dependent kinases (Cdks) and ubiquitin-mediated proteolysis of several regulatory proteins, including cyclins, the activators of Cdks (Morgan, 2007). This combined approach ensures that cell cycle events occur in the correct sequence and that the cell cycle is unidirectional.

4.1. Cyclin-dependent kinases and cell cycle control

Cyclin-dependent kinases (Cdks) are important Serine/Threonine protein kinases that have multiple regulatory roles during the eukaryotic cell cycle. Cdks are heterodimeric enzymes composed of a catalytic protein kinase subunit and a cyclin subunit (Pollard et al., 2004). The concentration of the kinase subunit is relatively constant, whereas the concentration of the cyclin subunit oscillates throughout the cell cycle (Figure 1.11). The cyclic accumulation and destruction of cyclins during interphase and mitosis, respectively, was the origin of their name (Evans et al., 1983). The primary mechanism of Cdk activation is the binding of a cyclin subunit. However, complete activation of most Cdks also requires phosphorylation of key residues in the activation loop of the kinase subunit by a Cdk-activating kinase (CAK) (Murray, 2004). The fully active enzymes can be turned off by various mechanisms. These mechanisms include degradation of cyclins by highly specific ubiquitin-mediated proteolysis (Peters, 2006), binding of Cdk inhibitory subunits (CKIs) (Morgan, 1997), and inhibitory phosphorylation of specific residues (Kellogg, 2003).

4.1.1. Cyclin-dependent kinases and cell cycle transitions

Distinct Cdk-cyclin complexes are formed at discrete windows during the cell cycle to regulate cell cycle transitions and important processes like DNA replication and mitosis (Figure 1.11). This regulation occurs primarily through the oscillations in cyclin concentration characteristic of actively dividing cells (Hochegger et al., 2008). Cyclins can be grouped into four categories corresponding to the cell cycle stage during which they accumulate: the G1 phase cyclin (cyclin D in vertebrates), the G1/S phase cyclin (cyclin E in vertebrates), the S phase cyclin (cyclin A in vertebrates) and the mitotic cyclin (cyclin B in vertebrates) (Figure 1.11). The oscillation of cyclin levels is regulated by temporally controlled gene expression, and by ubiquitin-mediated degradation via the proteasome. Ubiquitination is performed by the Skp1/Cul1/F-box protein (SCF) complex, in the case of interphase cyclins, or by the anaphase-promoting complex/cyclosome (APC/C), in the case of mitotic cyclins (Murray, 2004; Morgan, 2007). Another important level of control is the subcellular localization of the different cyclins. While cyclin E and A are present in the nucleus during interphase, cyclin B is cytoplasmatic in interphase and enters the nucleus upon mitotic entry (Murray, 2004). The physical localization of these cyclins correlates with their main functions during cell cycle. Another elegant mechanism to ensure directionality of the cell cycle is the fact that the initial G1 Cdk-cyclin complex activity promotes the expression of the S phase cyclins leading to the activation of S phase Cdk-cyclin complexes, which in turn inhibit the previous G1 Cdk-cyclin complexes (Murray, 2004; Morgan, 2007).

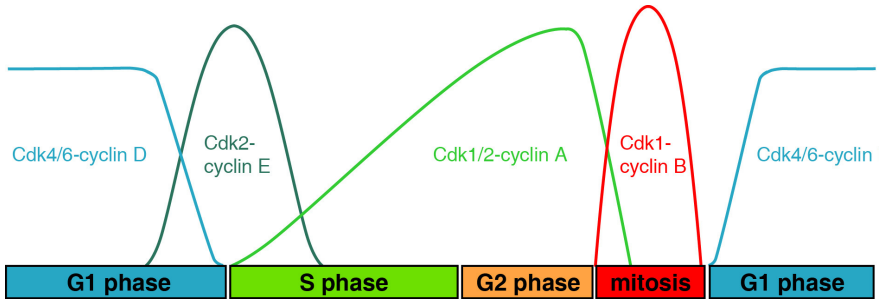

Figure 1.11. Classic model of cyclin-dependent cell cycle control in vertebrates. Cell cycle dependent oscillations of indicated cyclins are depicted. According to this model of cell cycle control, Cdk4-cyclin D and Cdk6-cyclin D regulate events in early G1 phase, Cdk2-cyclin E triggers S phase, Cdk2-cyclin A and Cdk1-cyclin A regulate the completion of S and G2 phases and entry into mitosis, and Cdk1-cyclin B is responsible for mitosis.

4.1.2. Evolution of the Cdk protein family

The number of Cdks is variable within eukaryotes, indicating that this kinase family evolved and expanded during eukaryotic evolution leading to the appearance of more specialized members (Morgan, 1997).

Yeasts, where Cdk was initially identified (Nurse, 1975), have a single Cdk that regulates the cell cycle transitions through its association with multiple stage-specific cyclins. In the budding yeast *Saccharomyces cerevisiae*, Cdc28 (the budding yeast Cdk) associates with three redundant G1 cyclins, Cln1-3, and with a family of six cyclins, Clb1-6, that control entry into S phase (Clb5 and 6) and mitosis (Clb1-4) (Nasmyth, 1996). In the fission yeast *Schizosaccharomyces pombe*, the mitotic functions of Cdc2 (the fission yeast Cdk) require a single cyclin, Cdc13, while the S phase functions involves predominantly Cig2 as well as Cig1 (Stern and Nurse, 1996).

In contrast, vertebrates have more than ten distinct Cdks. However, not all play direct roles in cell cycle progression. Cdk1 (or Cdc2) and Cdk2 are major players in cell cycle regulation. Cdk2 is activated by cyclin E at the beginning of S phase to initiate DNA synthesis and Cdk1 is activated by cyclin B at the end of

G2 phase to induce entry into mitosis. Cdk2 and Cdk1 also associate with cyclin A, during S and G2 phases, to promote S phase progression and participate in mitotic entry (Figure 1.11) (Hochegger et al., 2008). Cdk4 and Cdk6 are activated by cyclin D during G1 phase and are required for S phase entry as they activate the expression of genes required for DNA replication. Cdk7 associates with cyclin H and acts as a CAK, leading to activation of Cdk2 (Morgan, 1997). The other Cdks participate in diverse processes like transcription regulation (Cdk7-10) and neuronal differentiation (Cdk5) (Satyanarayana and Kaldis, 2009).

Although Cdk2, Cdk4 and Cdk6 play important roles during the vertebrate cell cycle, Cdk1 is sufficient to drive cell cycle progression in embryonic fibroblasts when these Cdks are not present, revealing they are not essential for cell cycle progression (Santamaría et al., 2007; Hochegger et al., 2008). This result shows that, like in yeast, cell cycle progression in vertebrates can be driven by a unique Cdk (Cdk1), suggesting that the basic mechanism of cell cycle regulation is highly conserved within all eukaryotes.

4.1.3. Substrate specificity of Cdks

Cdks control important processes throughout the cell cycle by phosphorylating a large number of substrates. These substrates are phosphorylated at Serine (S) or Threonine (T) residues in a preferred sequence context ([S/T*]-P-X-[K/R], where X is any amino acid) that is recognized by the active site of Cdks (Murray, 2004; Morgan, 2007). Since the majority of Cdks recognize this consensus sequence, the substrate specificity is conferred by the different cyclins. This explains how a single Cdk can control the entire cell cycle in yeast cells and in mouse embryonic fibroblasts. The cyclins can control Cdk substrate specificity by direct binding to the substrate or by directing Cdk activity to the subcellular compartment where the substrate is localized (Murray, 2004; Morgan, 2007).

Both cyclin A and cyclin E contain a hydrophobic binding pocket on their surface, that recognizes an RXL motif (where X is any amino acid). This motif is important to increase substrate specificity, especially when the substrate lacks the phosphorylation consensus sequence. RXL motifs are found in most but not all S phase substrates of Cdk2-cyclin A and Cdk2-cyclin E and in some CKI proteins (Morgan, 2007). Cyclin B binding to RXL motifs is weaker than cyclin A and cyclin E binding, due to sequence changes in its hydrophobic path (Brown et al., 2007). Cyclin B is less discriminatory in substrate recognition and controls Cdk substrate specificity largely through its subcellular localization. During interphase, cyclin B is actively exported from the nucleus blocking access to nuclear substrates.

4.1.4. Cdk activity regulates DNA replication

In eukaryotic cells, DNA replication is initiated at multiple specific regions called origins of replication. The activation of these replication origins occurs in two steps: a licensing step and an initiation step. Licensing occurs upon mitotic exit and consists of the formation of a specific protein-DNA complex called the pre-replicative complex (pre-RC). The pre-RC is formed via the ordered recruitment of the origin recognition complex (ORC), Cdc6, Cdt1 and the Mcm2-7 complex (Figure 1.12). The initiation step occurs at S phase onset and consists of activation of the pre-RC complex and in establishment of bidirectional replication forks. During this reaction a so-called CMG helicase complex (Cdc45-Mcm2-7-GINS) is formed (Figure 1.12), leading to the unwinding of double stranded DNA and the loading of DNA polymerases (Bell and Dutta, 2002; Tanaka and Araki, 2010).

Because some origins of replication initiate DNA synthesis early in S phase while others initiate DNA synthesis later in S phase, the licensing and initiation steps must occur separately in the cell cycle to avoid over-replication of some parts of the genome. Indeed, Cdks play a crucial role in separating these two

steps of DNA replication [Figure 1.12; (Arias and Walter, 2007; Tanaka and Araki, 2010)]. While the licensing step requires low Cdk activity, the initiation step needs high Cdk activity. This dual regulation of DNA replication by Cdks ensures that each replication origin is licensed and replicated only once during the cell cycle (Woo and Poon, 2003; Arias and Walter, 2007; Tanaka and Araki, 2010).

In budding yeast, low Cdk^{Cdc28} activity regulates DNA replication by activating the formation of pre-RC complexes in G1 phase, and high Cdk^{Cdc28} activity inhibits the pre-RC reassembly (Diffley, 2004; Arias and Walter, 2007). Cdks inhibit pre-RC assembly by directly phosphorylating and inhibiting each of the pre-RC components. ORC is inhibited by Cdk-mediated phosphorylation of Orc2 and Orc6 (Wilmes et al., 2004; Tanaka and Araki, 2010). Cdc6 is inhibited by Cdk at three distinct levels: first, Cdk phosphorylates Cdc6 and marks it for ubiquitin-mediated proteolysis via the SCF complex; second, Cdk activity inhibits Cdc6 transcription by blocking the transcription factor Swi5; finally, Cdk phosphorylation of Cdc6 induces its association with Cdc28-Clb2 complexes, blocking Cdc6 licensing activity (Arias and Walter, 2007; Tanaka and Araki, 2010). The Mcm2-7 complex, when phosphorylated by Cdks, is exported from the nucleus. This also leads to translocation of Cdt1 to the cytoplasm, due to its association with the Mcm2-7 complex (Diffley, 2004; Tanaka and Araki, 2010). In summary, budding yeast cells have several independent Cdk-mediated strategies to prevent pre-RC assembly during S, G2 and M phases.

In vertebrates, in addition to Cdk activity, other factors such as PCNA, $Cul4\text{-}Ddb1^{Cdt2}$ ubiquitin ligase, and geminin are required to control pre-RC assembly and inhibit over-replication (Diffley, 2004; Arias and Walter, 2007; Porter, 2008). The activity of these additional factors is regulated in a cell cycle-dependent manner. Therefore, Cdk activity likely prevents over-replication directly, by inhibiting some pre-RC components, and indirectly, by specifying cell cycle position. For instance, in mammalian cells, Cdt1 is degraded via the $Cul4\text{-}Ddb1^{Cdct2}$ ubiquitin ligase during S phase in a manner dependent on PCNA. Additionally, Cdt1 is targeted for destruction via the SCF complex during G2

through a Cdk2-cyclin A-mediated phosphorylation (Arias and Walter, 2007; Porter, 2008). Geminin binds and also inactivates Cdt1 thereby preventing pre-RC formation (Melixetian and Helin, 2004; Saxena and Dutta, 2005). Upon APC/C activation during mitotic exit geminin is degraded and Cdt1 is released to form new pre-RC complexes at replication origins. Therefore replication licensing and prevention of relicensing is not only controlled by Cdk mediated phosphorylation but also by cell cycle dependent destruction of an inhibitor of pre-RC formation.

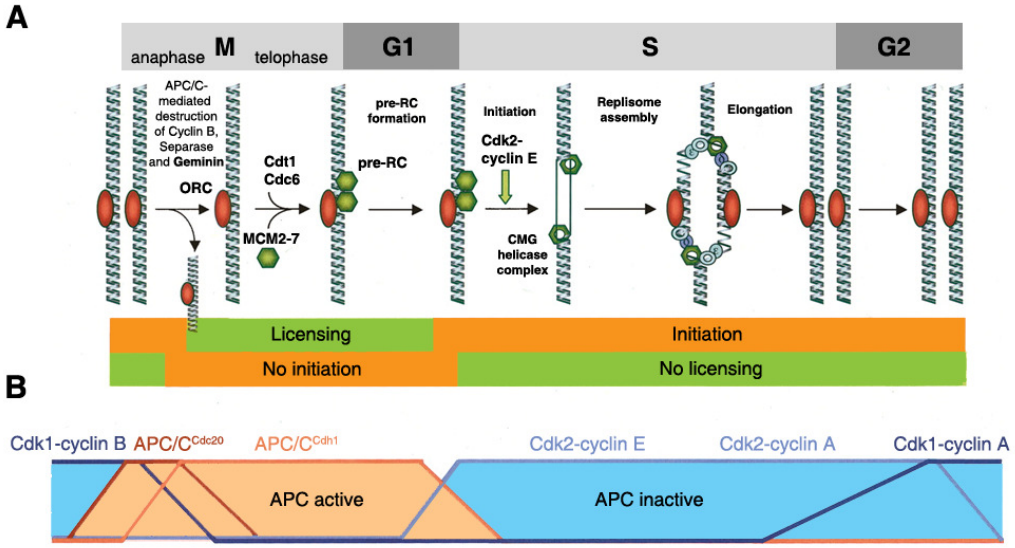

Figure 1.12. Two-step model for the cell cycle regulation of eukaryotic DNA replication. (A) The events that occur at origins of DNA replication at different stages of the cell cycle are shown. The green and orange bars indicate when in the cell cycle licensing and initiation are allowed. (B) Oscillations in APC/C and Cdk activity during the cell cycle are indicated. Adapted from Arias and Walter, 2007.

As mentioned above, Cdk activity not only controls the licensing step of DNA replication but also its initial step. In budding yeast Cdk promotes firing of the origins of replication through phosphorylation of the initiation-specific factors Dpb1, Sld2 and Sld3 (Tanaka and Araki, 2010). These proteins are required for

activation of the replicative helicase by recruiting GINS and Cdc45, which are part of the CMG helicase complex (Figure 1.12) (Tanaka and Araki, 2010). The CMG complex unwinds the DNA at replication origins and leads to the initiation of DNA synthesis by DNA polymerases (Araki, 2011). In contrast to the members of the pre-RC complex and the replication machinery, which are highly conserved among eukaryotes, the initiation factors Dpb11, Sld2 and Sld3 are not well conserved (Tanaka and Araki, 2010). However, all eukaryotes require Cdk activity for the initiation step of DNA replication and to prevent relicensing of origins of replication that already initiated replication.

4.1.5. Cdk activity controls centrosome duplication

The centrosome is another example of a major cellular component whose duplication and propagation is tightly cell cycle controlled. Centrosomes are composed of two centrioles that are surrounded by pericentriolar material (PCM) and form the major microtubule organizing centers (MTOCs) of animal cells, being crucial for cell division, motility and polarity (Bettencourt-Dias and Glover, 2007). Similar to chromosomes, centrosomes have to be duplicated and distributed to daughter cells during each cell cycle. The centrosome duplication cycle is coupled to the chromosome duplication cycle through the action of Cdk1, Cdk2 and Separase, the protease responsible for triggering anaphase by hydrolysing cohesin (Bettencourt-Dias and Glover, 2009). During centrosome duplication, one new centriole (daughter) forms orthogonally adjacent to each pre-existing centriole (mother), in a manner that resembles the template-mediated replication of DNA (Nigg and Stearns, 2011). There are four consecutive steps in the centrosome cycle: disengagement of the centrioles, formation of the daughter centrioles, elongation of the daughter centrioles, and separation of the centrosomes (Bettencourt-Dias and Glover, 2009; Nigg and Stearns, 2011). Disengagement of centrioles is coordinated with sister chromatid segregation during mitotic exit and acts as a licensing step for duplication in the

next cell cycle (Tsou and Stearns, 2006). This step requires Plk1 activity and APC/C-mediated degradation of securin that in turn activates separase, which occurs concomitantly with loss of Cdk1 activity (Tsou et al., 2009). Formation of the daughter centrioles is coordinated with DNA synthesis and both begin at the G1/S transition. Both duplication events require Cdk2-cyclin E activity (Hinchcliffe et al., 1999; Lacey et al., 1999; Tanaka et al., 2007) and an additional kinase: the Dbf4-dependent kinase (DDK) in the case of DNA replication, and Plk4 for centrosome duplication (Heller et al., 2011; Puklowski et al., 2011). Nucleophosmin (NPM/B23) is the only known target of Cdk2-cyclin E that is involved in the initiation of centrosome duplication (Okuda et al., 2000). NPM/B23 is associated with unduplicated centrosomes and dissociates through a Cdk2-cyclin E-mediated phosphorylation that occurs at the entry of S phase. This dissociation of NPM/B23 from the centrosomes is a critical step in the centrosome duplication process. During S and G2 phases, the two daughter centrioles elongate and progressively recruit PCM (centrosome maturation) (Bettencourt-Dias and Glover, 2007). During late G2, the centrosomes that contain two mature centrioles are separated to form the poles of the mitotic spindle during mitosis (Bettencourt-Dias and Glover, 2007).

In summary, centrosome and chromosome duplication are coupled and restricted to once per cell cycle through the action of Cdk2-cyclin E in S phase and APC/C during mitotic exit. In both cases the licensing of duplication, centriole disengagement in centrosome duplication and pre-RC assembly in DNA replication, occurs when Cdk activity is low by APC/C mediated destruction of securin and geminin, respectively. High Cdk2-cyclin E activity at the G1/S transition will trigger the initiation step and prevent the relicensing of DNA and centrosome duplication.

4.2. Ubiquitin-mediated proteolysis and cell cycle control

While Cdks and cyclins play crucial roles during the cell cycle, ubiquitin-mediated protein degradation is equally important to ensure unidirectionality of the cell cycle (King et al., 1996; Morgan, 2007). Importantly, Cdk activity is regulated, in part, by ubiquitin-mediated destruction of cyclins.

Targeting of specific substrates for ubiquitination results in their degradation by the 26S proteasome (Hochstrasser, 1995). Ubiquitin (Ub) is a small regulatory protein that, when attached to other proteins, labels them for proteasome-mediated degradation. The assembly of an ubiquitin chain requires the sequential action of three enzymes: the E1 ubiquitin-activating enzyme, the E2 ubiquitin-conjugating enzyme, and the E3 ubiquitin ligase (Hershko, 1997). While activation of the pathway by E1 and E2 are rather generic, most of the substrate specificity is conferred by the E3 ubiquitin ligase that mediates the final ubiquitin transfer onto the substrate, targeting it for proteolysis by the 26S proteasome. Because of their role in substrate specificity, the E3 ubiquitin ligases are the most important targets for regulation in the ubiquitin-mediated proteolysis system (Hershko and Ciechanover, 1998). Two related E3 ubiquitin ligase complexes, the SCF complex and the APC/C, play important roles in cell cycle regulation (Willems et al., 2004; Peters, 2006). APC/C is mainly active in mitosis and G1 phase and plays a crucial role in mitotic exit, while the SCF complexes are more versatile and have a variety of functions at many stages of the cell cycle (Vodermaier, 2004). Additionally, Cul3 and Cul4-based ubiquitin ligases play important roles in controlling Aurora B localization and in preventing DNA re-replication, respectively (Nishitani et al., 2006; Sumara et al., 2007).

4.2.1. Essential functions of the SCF complex during cell division

The SCF complex is composed of three core subunits: Skp1, Cul1, and an F-box protein that determines substrate specificity. The F-box proteins recognize specific phosphorylation motifs of substrates. Therefore, F-box proteins and the

phosphorylation status of substrates regulate SCF activity during the cell cycle. SCF complexes containing the F-box protein Skp2 are responsible for ubiquitin-mediated degradation of cyclins D and E and the Cdk inhibitors p21 and p27 at the G1/S phase transition (Nakayama and Nakayama, 2005; Sumara et al., 2008). Additionally, SCF^{Skp2} targets Orc1 and Cdt1 for degradation during S phase and G2 (Nakayama and Nakayama, 2005). Thus, SCF^{Skp2} complexes have an important role in regulating Cdk activity and in preventing DNA re-replication. The F-box proteins β-TrCP1 and Tome-1 promote SCF-dependent degradation of Wee1 (Watanabe et al., 2004; Smith et al., 2007), a kinase that inhibits Cdk1-cyclin B activity, thereby promoting entry into mitosis. The $SCF^{\beta\text{-}TrCP1}$ complex is also responsible for degradation of the APC/C inhibitor Emi1/Rca1 (early mitotic inhibitor/regulator of cyclin A) during prophase (Peters, 2006; Sumara et al., 2008).

4.2.2. Essential functions of the APC/C during cell division

The APC/C is a large multi-subunit complex whose activity is mainly regulated by 1) phosphorylation, 2) by binding of two activator proteins (Cdc20 and Cdh1), and 3) by binding of a inhibitory protein called Emi1 (Acquaviva and Pines, 2006; Peters, 2006). Cdc20 activates APC/C during mitosis while Cdh1 activates APC/C during anaphase and G1 phase.

APC/C^{Cdc20} complexes are formed following SCF-mediated degradation of Emi1, which occurs at the onset of mitosis. However, APC/C^{Cdc20} remains inactive until all kinetochores are attached in a bipolar manner to spindle microtubules through the action of the mitotic checkpoint complex (MCC) that is produced by unattached kinetochores (see also Section 2.4.1, Figure 1.8). Although APC/C^{Cdc20} is prevented from targeting most of its substrates by the MCC, APC/C^{Cdc20}-dependent destruction of cyclin A occurs during prometaphase before silencing of the mitotic checkpoint (Geley et al., 2001; den Elzen and Pines, 2001). This can be explained by the fact that cyclin A can bind Cdc20 with such

high affinity that it outcompetes MCC binding and inhibition (Di Fiore and Pines, 2010). Once all kinetochores are properly attached and the MCC concentration drops, APC/C^{Cdc20} is fully activated and triggers degradation of securin and cyclin B, which allows anaphase onset (Clute and Pines, 1999; Hagting et al., 2002). Securin is an inhibitor of separase, the protease responsible for cleavage of the Scc1 subunit of cohesin complex that holds sister chromatids together (Nasmyth et al., 2000; Waizenegger et al., 2000). Whereas degradation of securin leads to chromosome segregation, degradation of cyclin B leads to irreversible inactivation of Cdk1.

Following anaphase onset, loss of Cdk1 activity leads to the formation of APC/C^{Cdh1} complexes. APC/C^{Cdh1} promotes degradation of Cdc20, ensuring that APC/C is uniquely activated by Cdh1 after anaphase initiation (Peters, 2006). During mitotic exit, APC/C^{Cdh1} induces degradation of two important mitotic kinases, Plk1 and Aurora A (Lindon and Pines, 2004). Moreover, APC/C^{Cdh1} also targets geminin for degradation during mitotic exit, which enables licensing of DNA replication (McGarry and Kirschner, 1998). During G1 phase, APC/C^{Cdh1} ensures continued degradation of cyclin A and cyclin B until cells are committed to another round of DNA replication (Peters, 2006). Importantly, APC/C^{Cdh1} degrades the F-box proteins Skp2 and Tome-1 (Ayad et al., 2003; Wei et al., 2004), maintaining SCF inactive during G1 phase and allowing the accumulation of Cdk1 and Cdk2 inhibitors, cyclins D and E, and components of the pre-RC complex. APC/C^{Cdh1} is inactivated later at the G1/S transition to allow accumulation of substrates such as cyclins that are required for the initiation of DNA replication and subsequent entry into mitosis. Different mechanisms are in place to inhibit APC/C^{Cdh1}. First, Cdk2-cyclin A phosphorylates Cdh1, promoting its dissociation from the APC/C (Lukas et al., 1999; Kramer et al., 2000). Second, phosphorylated Cdh1 is targeted by the SCF complex (Benmaamar and Pagano, 2005), further limiting the activity of APC^{Cdh1}. Finally, in late G1 phase, Emi1 inhibits the activity of APC/C^{Cdh1} as a pseudo-substrate (Di Fiore and Pines, 2007).

Although APC/C and SCF play independent roles throughout the cell cycle, there is crosstalk between these two ubiquitin ligases. Indeed, they control the activity of each other by directing activators and inhibitors of each other for proteasome-mediated degradation (reviewed in Vodermaier, 2004).

4.3. Regulation of mitosis by mitotic kinases and phosphatases

Mitotic progression is under control of a wide range of kinases. Although the most prominent mitotic kinase is Cdk1, several other kinases are activated at the onset of mitosis to help control important mitotic events. These additional mitotic kinases include members of the Aurora and Polo-like kinase (Plk) families (Nigg, 2001; Ma and Poon, 2011).

Plk1 plays important roles in regulating mitotic entry, centrosome maturation, kinetochore-microtubule attachment, removal of cohesin from chromosome arms, chromosome segregation, spindle elongation and cytokinesis (Petronczki et al., 2008). Such multiplicity of functions is achieved through dynamic localization of Plk1. In prophase Plk1 associates with centrosomes, then becomes enriched at kinetochores in prometaphase and metaphase, followed by recruitment to the central spindle in anaphase, and finally accumulates in the midbody during late mitosis (Petronczki et al., 2008).

Aurora A and Aurora B also play important functions during mitosis. Although these two members of the Aurora kinase family have a very similar sequence and structure, they have distinct localizations and functions during mitosis (Carmena et al., 2009). Aurora A is localized at centrosomes and on the mitotic spindle, and regulates mitotic entry, centrosome maturation and separation as well as bipolar spindle assembly and stability (Barr and Gergely, 2007). Aurora B is a member of the chromosomal passenger complex and as such it localizes to the inner centromere until metaphase and then transfers to the spindle midzone and finally to the midbody in late mitosis and cytokinesis, respectively. Aurora B regulates spindle assembly, removal of cohesin between

chromosome arms and cytokinesis (Ruchaud et al., 2007; Carmena et al., 2009). Another well characterized role of Aurora B is the promotion of chromosome biorientation by correcting mis-attachments until the bioriented chromosome is under tension (Carmena et al., 2009).

As discussed above, a large number of substrates are phosphorylated by these mitotic kinases to perform important functions during mitosis. Both kinases and phosphatases modulate the phosphorylation status of the various mitotic substrates (Wurzenberger and Gerlich, 2011). Cdc25 phosphatases are highly conserved among eukaryotes and play a crucial role in mitotic entry by removing the inhibitory phosphorylation on Cdk1-cyclin B complexes (Bollen et al., 2009). Phosphatases are also vital for mitotic exit by dephosphorylation of the substrates of the different mitotic kinases.

In budding yeast, the mitotic exit process is well characterized and requires activation of the phosphatase Cdc14 (Queralt and Uhlmann, 2008). However, in most other eukaryotes Cdc14 is dispensable for mitotic exit, indicating, that contrary to mitotic kinases that are highly conserved within eukaryotes, the function of mitotic exit phosphatases has diverged. The function of phosphatases during mitotic exit in animal cells is poorly characterized, but recent studies highlight the importance of the PP1 and PP2A phosphatase families. PP2A has a key role in dephosphorylating Cdk1 substrates during mitotic exit and its activity is, at least in part, regulated by Greatwall kinase (Wurzenberger and Gerlich, 2011). An additional role of PP2A consists of protecting centromeric cohesion until anaphase onset by dephosphorylation of the cohesion subunit SA2, which when phosphorylated by Plk1 promotes removal of cohesin complexes from chromosomes (Rivera and Losada, 2006). PP1 does not seem to directly dephosphorylate Cdk1 substrates (Ferrigno et al., 1993), but it appears to counteract Aurora B phosphorylation, to stabilize kinetochore-microtubule attachment during metaphase and to regulate mitotic chromosome decondensation (Vagnarelli et al., 2006).

5. Aims of this thesis

Centromeres form the site of chromosome attachment to microtubules during mitosis and meiosis and are therefore crucial for proper chromosome segregation. The identity of these chromosomal loci is maintained epigenetically by nucleosomes containing the histone H3 variant CENP-A. As such, CENP-A chromatin must be maintained and replicated during each round of cell division. Propagation of CENP-A chromatin is uncoupled from DNA replication and initiates only upon mitotic exit. Gaining insight into how CENP-A chromatin is maintained throughout cell divisions is crucial for our understanding of the mechanisms of cell division and epigenetic inheritance.

At the onset of the research described in this thesis, only a few proteins were described to have a role in CENP-A assembly. Factors that bind CENP-A specifically and affect its deposition into centromeric chromatin had not yet been identified. In Chapter 2 of this thesis we focus on characterizing new proteins that bind directly to CENP-A and/or have a role in CENP-A assembly. To this end we developed a powerful SNAP-based approach to assess CENP-A assembly directly and determine the role of novel factors in this process. During the course of this thesis work we and several other groups made significant progress in this area. I have integrated these recent findings and propose a comprehensive model on how centromere propagation is achieved.

In addition to identifying the factors that mediate CENP-A deposition it is also important to further understand the cell cycle control mechanism that restricts CENP-A assembly to a specific time window during late mitosis/early G1 phase. The requirement of mitotic exit for CENP-A assembly ensures a direct coupling of centromere inheritance with cell cycle progression and indicates that some mitotic event may be critical to activate the CENP-A assembly process. In Chapter 3 of this thesis we sought to identify the mitotic trigger that initiates and restricts centromere propagation to this unique cell cycle window. We determined

that the principal molecular signal that temporally controls CENP-A assembly is the inactivation of cyclin-dependent kinase 1 (Cdk1) and Cdk2.

In Chapter 4, we advanced on the molecular mechanism through which Cdk1 and Cdk2 control G1 phase timing of CENP-A assembly. The results described in this chapter led us to propose a model in which Cdk1 and Cdk2 inhibit CENP-A assembly during most of the cell cycle through phosphorylation of one or more CENP-A assembly factors preventing their centromere targeting.

In summary, my thesis work focused on identifying and characterizing new CENP-A assembly factors and in determining the detailed molecular mechanism that controls and restricts CENP-A assembly to late mitosis/early G1 phase of the cell cycle.

In Chapter 5 the results and conclusion from my thesis work are integrated with recent published findings on the CENP-A assembly process and presented in a general overview which includes ongoing work and an outlook to the future.

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Chapter 2 – Dissecting the CENP-A assembly pathway

Abstract

Centromeres are specialized chromosomal domains that ensure proper chromosome segregation during mitosis. CENP-A, a histone H3 variant, present exclusively at centromeres, functions as an epigenetic mark responsible for centromere function and propagation. Unlike canonical histones, that are assembled during DNA replication throughout chromatin, the assembly of nascent CENP-A into nucleosomes is restricted to early G1 phase of the cell cycle. Elucidating how CENP-A chromatin is propagated across cell divisions is crucial for our understanding of the broad mechanisms of cell division and epigenetic inheritance. To understand the mechanisms that selectively target CENP-A to centromeres upon mitotic exit we developed a powerful approach, based on siRNA-mediated depletion and pulse labeling of proteins, to identify new CENP-A assembly factors. Using this approach we confirmed the role of the Mis18 complex in CENP-A assembly and, moreover, we showed direct evidence that this protein complex is target to the centromeres prior to CENP-A, possibly licensing the centromeric chromatin for subsequence CENP-A assembly. Additionally, we revealed that CENP-N and CENP-C, two proteins that bind directly to CENP-A nucleosomes, as well as CENP-T, are involved in CENP-A incorporation into centromeres. As these proteins depend on CENP-A for their centromere localization, we propose they form the basis of an epigenetic feedback loop responsible for propagation of centromeric chromatin. Concurrently with this work, other studies identify other novel players of the CENP-A assembly pathway, leading us to propose a comprehensive model on how centromeric chromatin is propagated across cell divisions.

Introduction

The centromere is a unique chromosomal locus that is required for the assembly of the kinetochore, the structure to which spindle microtubules attach during mitosis and meiosis to allow accurate chromosome segregation (Allshire and Karpen, 2008; Verdaasdonk and Bloom, 2011). Centromere function is remarkably conserved between species, yet centromeric DNA sequences are highly divergent and are neither required nor sufficient for centromere identity in most eukaryotes, with the exception of budding yeast (Cheeseman et al., 2002; Malik and Henikoff, 2009). Instead, centromeres are thought to be epigenetically specified (Vafa and Sullivan, 1997; Warburton et al., 1997; Warburton, 2004). In all eukaryotes, centromeric regions differ from the rest of the chromatin in that they contain specialized nucleosomes, in which the canonical histone H3.1 is replaced by a unique histone H3 variant that was identified in humans as CENP-A (Palmer et al., 1987; Palmer et al., 1991; Yoda et al., 2000). This protein is the primary candidate for the epigenetic mark that specifies centromere identity due to its specific assembly into centromeric nucleosomes and its capacity to nucleate the formation of the entire centromere/kinetochore complex (Ahmad and Henikoff, 2002; Liu et al., 2006; Black et al., 2007). Supporting this idea, CENP-A is necessary and sufficient to nucleate a heritable and functional centromere in both human and fly cells (Barnhart et al., 2011; Mendiburo et al., 2011; Olszak et al., 2011).

Despite the discovery of the central role of CENP-A in maintaining centromere identity it is unclear how this epigenetic mark is itself inherited and propagated across cell divisions. An important advance on this question was the identification of a discrete cell cycle window during which CENP-A is assembled into chromatin (Jansen et al., 2007; Schuh et al., 2007). Unlike canonical histones, which are assembled during S phase, CENP-A is redistributed between the two sister chromatids during DNA replication, and its assembly into centromeric chromatin is delayed until late telophase/early G1 phase (Jansen et

al., 2007; Hemmerich et al., 2008; Dunleavy et al., 2011). Therefore, it is likely that the factor(s) regulating the unique timing of CENP-A assembly are centromere-localized within this unique cell cycle window. Indeed, a complex of proteins was identified that appears to fulfill this requirement. The founding member of this complex, Mis18, was initially identified in fission yeast and was shown to be required for CENP-A^{Cnp1} targeting to the centromere (Hayashi et al., 2004). This protein accumulates at centromeres upon mitotic exit and its temporal localization pattern is conserved in a complex of human proteins that include the Mis18 homologs Mis18α and Mis18β as well as the myb-domain-containing protein, Mis18BP1^{HsKNL2} (Fujita et al., 2007; Maddox et al., 2007). Depletion of any of these proteins causes a dramatic reduction of CENP-A levels at the centromere. However, none of these proteins appear to directly interact with CENP-A. In fission yeast, Mis18 forms a complex with Mis16 and both function as upstream factors of the CENP-A^{Cnp1} assembly pathway (Hayashi et al., 2004). The human homologues of Mis16, called RbAp46 and RbAp48, are also required for CENP-A localization at the centromere (Hayashi et al., 2004; Fujita et al., 2007; Dunleavy et al., 2009). Both of these proteins are members of the chromatin assembly factor 1 (CAF-1) complex and RbAp48 is also part of the HIRA complex, which are responsible for the assembly of the canonical histone H3.1 and the H3.3 variant, respectively (Verreault et al., 1996; Tagami et al., 2004). RbAp48 was also found in CENP-A prenucleosomal complexes (Dunleavy et al., 2009), indicating that it serves as a general chaperone involved in the assembly process of all histone H3 variants, possibly through a direct interaction with histone H4. Additionally, the fission yeast inner centromere protein, Mis6 is also required for CENP-A^{Cnp1} localization at the centromere (Takahashi et al., 2000). Although this protein is localized to centromeres throughout the cell cycle, it appears to act primarily before or at the onset of S phase, which is consistent with the time during which CENP-A is assembled into fission yeast centromeres (Saitoh et al., 1997). The human homologue of Mis6, CENP-I, forms a complex with CENP-H and CENP-K and these proteins, as well as CENP-M, have also

been implicated in CENP-A assembly (Okada et al., 2006). Although these proteins are part of the CENP-A nucleosome associated complex, a direct interaction between them and CENP-A has not been found (Foltz et al., 2006; Carroll et al., 2009). Members of the human Mis12 complex, which have a central role in kinetochore assembly, also influence CENP-A localization at centromeres (Kline et al., 2006).

In summary, a considerable number of proteins have been implicated in CENP-A assembly but factors that bind CENP-A specifically and affect its deposition into centromeric chromatin have not yet been identified. During the course of this thesis we and several other groups made significant progress on resolving this problem. In this chapter we will review and discuss these recent findings including the identification of HJURP and Scm3, which might function as specific chaperones that deliver CENP-A into human and fission yeast centromeres, respectively (Dunleavy et al., 2009; Foltz et al., 2009; Pidoux et al., 2009; Williams et al., 2009; Shuaib et al., 2010). To further understand how centromeric chromatin is established we developed a powerful SNAP-based pulse labeling approach to identify new CENP-A assembly factors. We confirmed the role of the Mis18 complex in CENP-A assembly, and demonstrated the involvement of three structural centromere components in loading CENP-A into centromeric chromatin. Our results reinforce the idea that CENP-A assembly occurs in distinct steps which allows multiple levels of regulation.

Material and Methods

Cell lines and constructs

HeLa cells and their derivatives were cultured in DMEM medium supplemented with 10% newborn calf serum, 2 mM L-Glutamine, 100U/mL Penicillin and 100 µg/mL Streptomycin (all from Gibco), at 37°C and 5% CO_2. In this chapter we used HeLa cell lines stably expressing LAP-(GFP)-CENP-N (referred to as GFP-CENP-N throughout this chapter) [gift from D. Foltz, University of Virginia, Charlottesville, VA; (Foltz et al., 2006)] or CENP-A-SNAP (Jansen et al., 2007). A HeLa cell line stably expressing both CENP-A-SNAP and LAP-(GFP)-Mis18α was generated using the stable cell line expressing CENP-A-SNAP. A construct containing LAP-(GFP)-Mis18α (a gift from I. Cheeseman, MIT, Cambridge, MA) was stably integrated into this cell line using Moloney murine leukemia retroviral delivery as previously described (Shah et al., 2004). Cells stably expressing CENP-A-SNAP and LAP-(GFP)-Mis18α (referred to as GFP-Mis18α throughout this thesis) were selected using 5 µg/mL of Blasticidin S (Invitrogen) and 1,5 µg/mL of Puromycin (Calbiochem), and single-cell sorted in a MoFlo High-Speed Cell Sorter (Beckman Coulter, USA). The resulting monoclonal lines were expanded and selected by fluorescence microscopy for expression level and localization.

Cell synchronization

HeLa cells were synchronized by a double Thymidine block. Cells were treated with 2 mM of Thymidine (Sigma) for 17 hours , washed twice in medium and released in medium containing 24 µM of Deoxycytidine (Sigma) for 9 hours. Subsequently, cells were treated again with Thymidine for 16 hours, and finally released into medium containing Deoxycytidine and assayed.

siRNA transfection

All siRNAs were obtained from Dharmacon. Smart pools were used to deplete CENP-N, CENP-T, CENP-C, Mis18BP1^{HsKNL2} and HJURP. CENP-A, Mis18α and GAPDH were depleted using single siRNA oligos. All the oligos described in this chapter are listed in table 2.1.

Table 2.1. List of siRNA oligos used in chapter 2

Target	Sequence
CENP-A	5'-ACAGUCGGCGGAGACAAGG-3'
GAPDH	5'-CAACGGAUUUGGUCGUAUU-3'
Mis18α	5'-CAGAAGCUAUCCAAACGUG-3'
CENP-N	5'-GUAAAUUUCCGACAGAGAA-3'
	5'-CUACCUACGUGGUGUACUA-3'
	5'-GAUAUUACCGAAAUGAAGA-3'
	5'-CCAGAAAGUUUGGGAUGUU-3'
CENP-T	5'-CAAGAGAGCAGUUGCGGCA-3'
	5'-GACGAUAGCCAGAGGGCGU-3'
	5'-AAGUAGAGCCCUUACACGA-3'
	5'-CGGAGAGCCCUGCUUGAAA-3'
CENP-C	5'-GCGAAUAGAUUAUCAAGGA-3'
	5'-GAACAGAAUCCAUCACAAA-3'
	5'-CGAAGUUGAUAGAGGAUGA-3'
	5'-UCAGGAGGAUUCGTGAUUA-3'
HJURP	5'-GCACGAGGGACCAUCAGUU-3'
	5'-UGGAGUGUCUACAGAUAAA-3'
	5'-GUGACACCCUCGAAGUAUU-3'
	5'-UGGUUAAUUUCUCCUGUAA-3'
Mis18BP1^{HsKNL2}	5'-CUACAGGAAUGGAUGAUUA-3
	5'-CAACAAGGAUGGAUUAAAA-3'
	5´-UCAGUUGGCUAAACAAAUU-3'
	5'-GAUGAACGUGACUUACUUA-3'

The siRNA tranfections were performed in 24 well plates. For each condition, 60 pmoles of siRNAs and 3µL of Oligofectamine (Invitrogen) were separately incubated in 50 and 12 µL Optimem, respectively, for 5 minutes, after which they were mixed and incubated for an additional 20 minutes. The mix was supplemented with an additional 500 µL of Optimem (Gibco) and cells were incubated with the transfection mix for 4 hours, after which they were supplemented with fetal bovine serum (FBS; Gibco) to a final concentration of 10%. In the following day the mixture was replaced by fresh complete culture medium.

SNAP quench-chase-pulse labeling

HeLa cells expressing CENP-A-SNAP were pulse labeled by addition of 2 µM BTP (Covalys) in growth medium for 30 minutes at 37ºC and 5% CO_2 for irreversible, nonfluorescent labeling of the preexisting CENP-A-SNAP pool. We refer to this step as "quench". Following quenching, cells were chased for 7 hours to allow synthesis of new and unlabeled CENP-A-SNAP. This new pool was subsequently labeled with 2 µM TMR-*Star* (Covalys) in growth medium for 15 minutes at 37ºC, 5% CO_2, thereby fluorescently and specifically labeling the nascent CENP-A-SNAP pool. After each labeling step (nonfluorescent and fluorescent), cells were washed twice with medium and reincubated at 37ºC to allow excess SNAP substrate to be released from cells. After 30 minutes, cells were washed again once in medium.

Immunofluorescence

HeLa cells were grown on glass coverslips [thickness: 1.5, coated with poly-L-Lysine (Sigma)] and fixed with 4% formaldehyde (Thermo Scientific) for 10 minutes. Cells were extracted after fixation and processed for immunofluorescence using standard procedures. Cells were stained with anti-cyclin B1 (1:50; sc-245, Santa Cruz), anti-CENP-T [1:1000; gift from D. Foltz,

University of Virginia, Charlottesville, VA; (Barnhart et al., 2011)], anti-CENP-C (1:10000; gift from D. Foltz, University of Virginia, Charlottesville, VA), anti-α-tubulin (1:2500; clone YL1/2, Serotec) and anti-HA (clone HA11, Covance) at the concentration of 1 µg/mL. Secondary antibodies (Cy5- or FITC-conjugated anti-mouse, FITC- or Cy5-conjugated anti-rabbit and Cy5-conjugated anti-rat) were obtained from Jackson Immunoresearch Laboratories. Cells were stained with DAPI (4',6-diamidino-2-phenylindole; Sigma) before mounting in ProLong (Invitrogen) or Mowiol (Sigma).

Immunoblotting

Extracts of 10^5 HeLa cells were separated in a 12% SDS-PAGE gels and transferred to Hybond PVDF membranes (GE Healthcare) using standard procedures. Blots were probed with anti-GFP [gift from D. Foltz, University of Viginia, Charlottesville, VA; (Foltz et al., 2009)] at 1:2000 dilution and anti-CENP-N at 1:200 dilution [gift from A. Straight, Stanford University, Palo Alto, CA; (Carroll et al., 2009)]. Anti-rabbit HRP-conjugated secondary antibodies were purchased from Jackson Immunoresearch Laboratories.

Microscopy

Widefield fluorescence microscopy was performed using a DeltaVision Core system (Applied Precision) that controls an inverted microscope (Olympus, IX-71), coupled to a Cascade2 EMCCD camera (Photometrics). 512 by 512 pixel images were collected at 1x binning using a 100x, 1.4 NA oil immersion objective (UPlanSApo) at 0,2 µm axial sections spanning the entire nucleus (typically 30 to 35 sections). Digital images quantified in Figure 2.4 were captured using a DeltaVision RT system (Applied Precision) controlling an interline charge-coupled device camera (Coolsnap, Roper) mounted on an inverted microscope (Olympus, IX-70). 512 by 512 pixel images were collected at 1x binning using a 40x oil objective at 0,2 µm axial sections. Quantified images were acquired using the

same exposure conditions for each fluorescent channel. TRITC, FITC and Cy5 images of a uniformly slide were automatically flatfield- and camera-noise-corrected using softWoRx (Applied Precision).

For time-lapse imaging, cells were seeded in an 8 well chambered coverglass (LabTek) and maintained at 37ºC in CO_2 independent media (Leibovitz; Gibco) supplemented with 10% FBS and 2 mM L-Glutamine. Cells were imaged every 10 minutes during 16 hours and 4 axial sections with 2 µm intervals were taken using a 100x, 1.4 NA oil immersion objective.

All images presented are maximum intensity projections of deconvolved pictures.

Fluorescence Quantification

Centromeric TMR-*Star* fluorescence intensity was quantified using *CRaQ*, a macro specifically developed for ImageJ (NIH). For specific details about methods and parameters of *CRaQ* see Bodor et al., 2012. Standard parameter settings for *CRaQ* were optimized as follows: square size = 7; minimum circularity = 0.95; maximum feret's diameter = 7; minimum centromere size = 4; maximum centromere size = 35; threshold factor = 1.1; chromatic aberration correction = (0,0). These settings were used for all analyses, with the following exception: *CRaQ* analysis of the data presented in Figure 2.4 was done using square size = 5. In figure 2.5 the centromere fluorescence intensity was quantified manually as described by Hoffman et al. (Hoffman et al., 2001).

Results

Mis18 complex is targeted to centromeres prior to CENP-A and is required for centromere assembly

Centromere targeting of the Mis18 complex, which includes Mis18α, Mis18β and Mis18BP1^{HsKNL2}, has been implicated as a priming step of CENP-A assembly (Fujita et al., 2007; Maddox et al., 2007). However, thus far, what constitutes "priming" has been poorly defined. To further assess the role of the Mis18 complex in the establishment of CENP-A chromatin, we developed an assay combining a gene down regulation approach with SNAP technology. This fluorescent pulse-labeling technique allows for the distinction between assembled (old) and pre-assembled (newly synthesized) CENP-A (Jansen et al., 2007). Using siRNA, we knocked down two members of the Mis18 complex, Mis18α and Mis18BP1^{HsKNL2}, and examined the effect on CENP-A assembly in HeLa cells stably expressing CENP-A-SNAP at near endogenous levels. Following siRNA transfection, we performed quench-chase-pulse labeling to visualize only nascent CENP-A-SNAP. To this end, cells were synchronized at the G1/S boundary by double Thymidine block. At this stage, the pre-existing pool of CENP-A-SNAP was labeled with a cell-permeable, nonfluorescent substrate (BTP) followed by release into S phase. During S phase, a nascent pool of CENP-A-SNAP was produced which was specifically labelled with a fluorescent substrate (TMR-*Star*) at the end of S phase (Figure 2.1 A). To test whether Mis18α and Mis18BP1^{HsKNL2} depletions have an effect on CENP-A assembly, we measure the centromeric levels of nascent CENP-A-SNAP (CENP-A TMR-*Star*) in cells depleted of each of these proteins. Depletion of GAPDH and CENP-A were used as negative and positive controls, respectively. Consistent with previous studies, we observed a defect in assembly of nascent CENP-A upon siRNA depletion of Mis18α or Mis18BP1^{HsKNL2} [Figure 2.1 B, C and (Fujita et al., 2007; Maddox et al., 2007)].

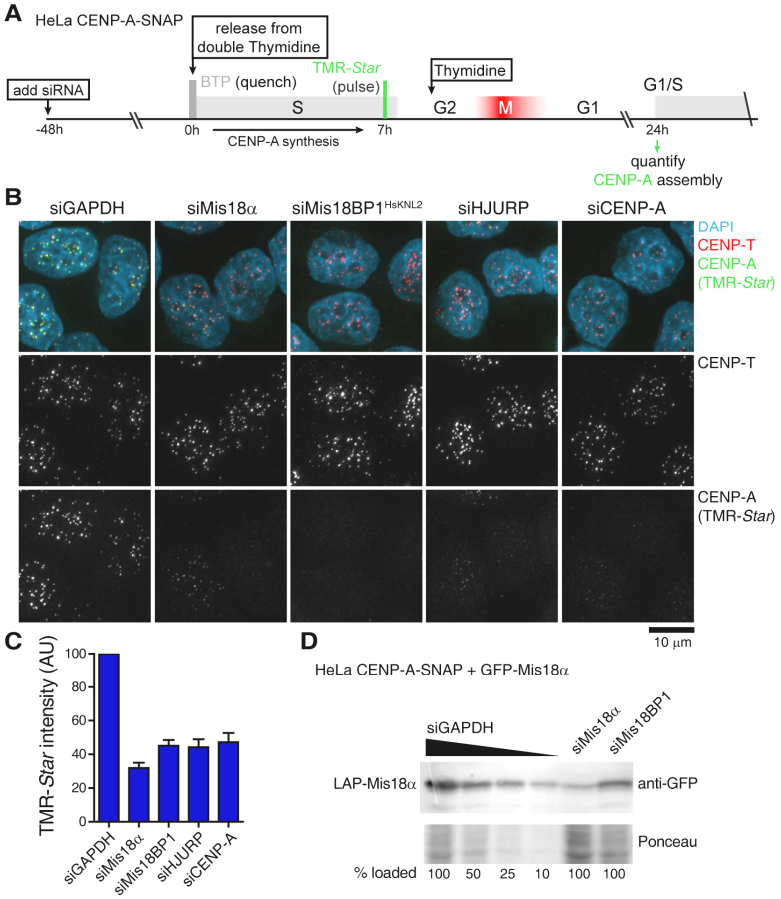

Figure 2.1. Mis18α and Mis18BP1^{HsKNL2} are required for CENP-A assembly. (A) HeLa cells expressing CENP-A-SNAP were transfected with siRNAs against indicated targets (GAPDH serves as negative control; CENP-A and HJURP serve as positive controls) and synchronized by double Thymidine block combined with SNAP quench-chase-pulse labeling. Cells were cycled into the next cell cycle and collected in Thymidine at the next G1/S boundary (t = 24 hours). (B) Cells were imaged and counterstained for CENP-T and with DAPI to indicate centromeres and DNA, repectively. (C) TMR intensity was quantified in more than 1200 centromeres per condition. Mean and SEM of 3 replicates of each condition are shown. (D) HeLa cells expressing both CENP-A-SNAP and GFP-Mis18α were transfected with siRNAs and synchronized as in A, followed by processing for SDS-PAGE and immunoblotting. Fraction of cells loaded is indicated for each condition. Efficiency of depletion of Mis18α and Mis18BP1^{HsKNL2} is assessed by GFP-Mis18α protein levels using anti-GFP antibodies [Mis18α and Mis18BP1^{HsKNL2} are interdependent (Fujita et al., 2007)], and Ponceau staining was used as a loading control.

Note that partial depletion of these proteins (to or below 50% of unperturbed levels; Figure 2.1 D) is sufficient to impair CENP-A assembly, indicating that these proteins are rate limiting for CENP-A assembly (Figure 2.1 B and C). These results validate the use of SNAP labeling in combination with siRNA mediated depletion as a powerful approach to analyse defects in CENP-A assembly and, therefore, to indentify new CENP-A assembly factors. Indeed, this strategy was used to demonstrate that HJURP, the CENP-A specific chaperone, is required for assembly of newly synthesized CENP-A (Foltz et al., 2009).

The Mis18 complex has been reported to target to centromeres during late anaphase (Fujita et al., 2007; Maddox et al., 2007), while CENP-A assembly appears to occur later, during late telophase/early G1 phase (Jansen et al., 2007; Hemmerich et al., 2008). Here, using a double tagged cell line expressing CENP-A-SNAP and GFP-Mis18α, we demonstrated in a direct manner the temporal disconnection between Mis18α and CENP-A targeting to centromeres (Figure 2.2 A and B). All anaphase/telophase cells analyzed (n=10) were positive for GFP-Mis18α but negative for newly synthesized CENP-A-SNAP (CENP-A TMR-*Star*). In contrast, in cells in early G1 phase (midbody positive; n=25) both proteins were observed at centromeres.

We confirmed the timing of Mis18α localization by live cell imaging of HeLa cells stably expressing GFP-Mis18α. GFP signals of Mis18α were observed in the nucleolus in G2 cells, and became dispersed upon nuclear envelope breakdown. In late anaphase, GFP-Mis18α was recruited to centromeres and its centromeric localization was lost during mid G1 (Figure 2.3 A and B). The duration of Mis18α retention at centromeres was variable among different cells and ranges from 2 hours and 30 minutes (Figure 2.3 A and Fujita et al., 2007) to 7 hours (Figure 2.3 B). This variation is likely caused by differences in expression levels of Mis18α, which determine in part the detection threshold by fluorescence microscopy. If we consider the loss rate of Mis18α from the centromere to be independent of Mis18α levels, then initial levels of Mis18α would determine the time this protein remains at the centromere. Consistently, in cells expressing

higher levels of Mis18α, the centromeric signals of this protein remained visible for a longer period (Figure 2.3 B).

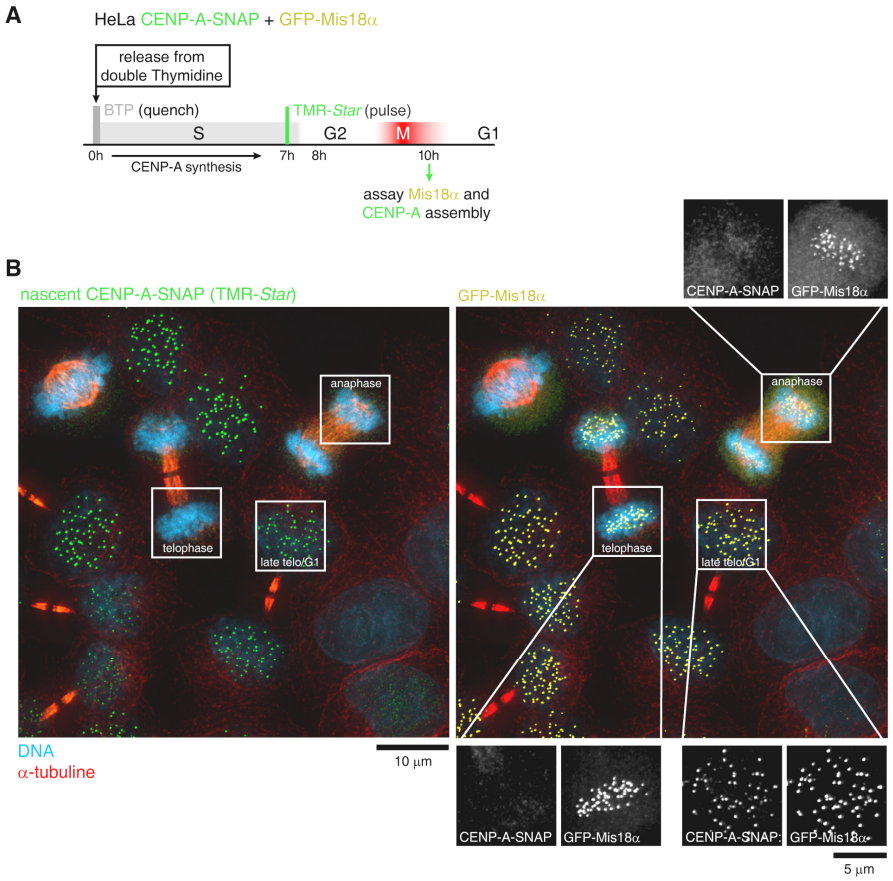

Figure 2.2. Mis18α targets to centromeres prior to CENP-A assembly. (A) HeLa cells stably expressing both CENP-A-SNAP and GFP-Mis18α were synchronized at the G1/S boundary by double Thymidine block. At this stage, the pre-existing pool of CENP-SNAP was quenched with BTP. An S phase synthesized pool of CENP-A-SNAP was subsequently pulse labeled 7 hours after release from Thymidine. Cells were fixed 10 hours after Thymidine release and counterstained for α-tubulin and with DAPI to visualize microtubules and DNA, respectively. (B) A single four channel field is shown as two separate images with TMR-*Star*-labeled nascent CENP-A-SNAP in green and GFP-Mis18α in yellow. Grayscale blowups show that Mis18α and CENP-A are targeted to centromeres at different times: Mis18α in anaphase and nascent CENP-A in late telophase/early G1 phase (see also Silva and Jansen, 2009).

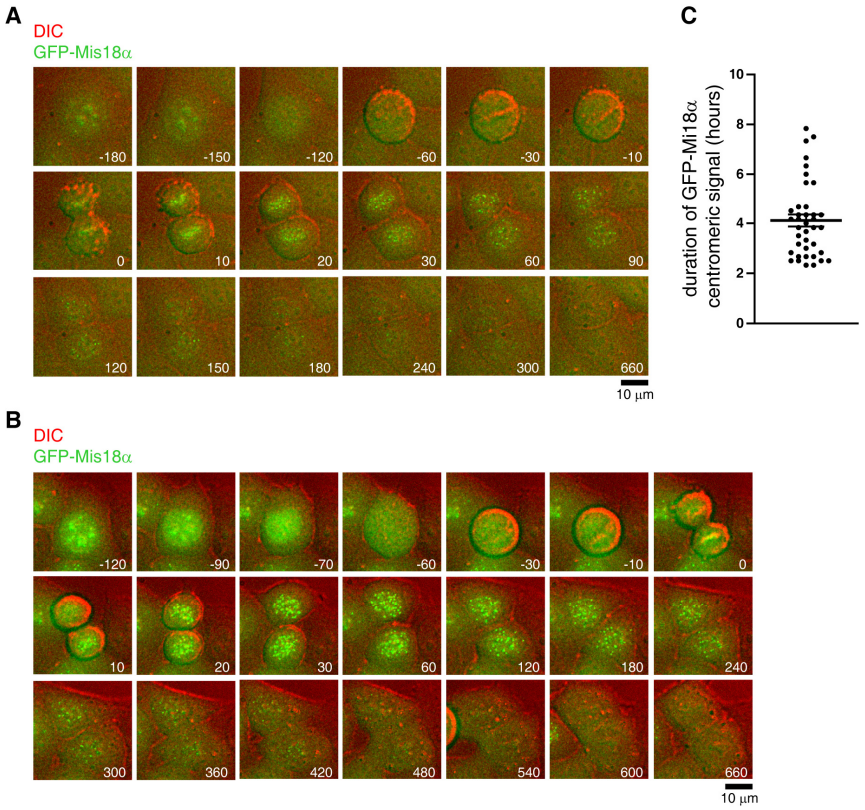

Figure 2.3. Mis18α assembles at centromeres in anaphase and dissipates in mid G1 phase. Still images from a time-lapse series of HeLa cells stably expressing GFP-Mis18α (green). (A) In cells expressing lower levels of GFP-Mis18α, the centromeric signals were observed for 2 hours. (B) In cells expressing higher levels of GFP-Mis18α, the centromeric signals were observed for up to 7 hours. (C) Scatter plot representing the duration time (in hours) of GFP-Mis18α centromeric signals on a per cell basis. The average duration of GFP-Mis18α at centromeres is approximately 4 hours (n=40).

Mis18α has a localization pattern very similar to CENP-A: they both accumulate at the nucleolus prior to mitosis and both target to centromeres upon mitotic exit, Mis18α during late anaphase and CENP-A during late telophase/early G1. Once assembled into centromeric chromatin, CENP-A is stably maintained and survives even throughout S phase and mitosis, when chromatin suffers chromatin disruption and compaction, respectively (Jansen et al., 2007; Dunleavy et al., 2011). Conversely, the Mis18 complex is lost from

chromatin during mid G1, suggesting it is not part of a stable chromatin complex. This indicates that, while crucial for the initial steps of CENP-A assembly, this complex is unlikely to be involved in maintaining CENP-A in subsequent phases of the cell cycle.

Structural components of the centromere affect CENP-A assembly

The constitutive centromere associated network (CCAN) is large group of centromere proteins arranged in subcomplexes that are present at the centromere throughout the cell cycle (Cheeseman and Desai, 2008; McAinsh and Meraldi, 2011; Perpelescu and Fukagawa, 2011). The CCAN is crucial for kinetochore assembly but has also been implicated in the establishment and propagation of CENP-A chromatin. CENP-H, CENP-I, CENP-K, and CENP-M have been shown to be required for the assembly of newly synthesized GFP-CENP-A in vertebrate centromeres (Okada et al., 2006). In *D. melanogaster* and in *X. laevis*, CENP-C is also required to recruit nascent CENP-A to the centromeres (Erhardt et al., 2008; Moree et al., 2011). Here, we analyzed the role of other CCAN components in the CENP-A assembly pathway in mammalian cells.

We transfected HeLa cells stably expressing CENP-A-SNAP with siRNA oligos against three members of the CENP-A nucleosome associated complex (CENP-C, CENP-N and CENP-T) and tested, by quench-chase-pulse labelling, whether their depletion reduced the levels of nascent CENP-A-SNAP loaded at centromeres (Figure 2.4 A). We measured the knockdown efficiency of CENP-C and CENP-T by immunostaining followed by quantification of the centromeric levels of these proteins in comparison to the levels following depletion of a GAPDH control. In the case of CENP-N, we used a cell line stably expressing GFP-CENP-N and quantified GFP levels at the centromere after depletion of CENP-N or GAPDH (Figure 2.4 C). We showed that depletion of any of these three proteins caused a defect on CENP-A loading (Figure 2.4 B, left graph).

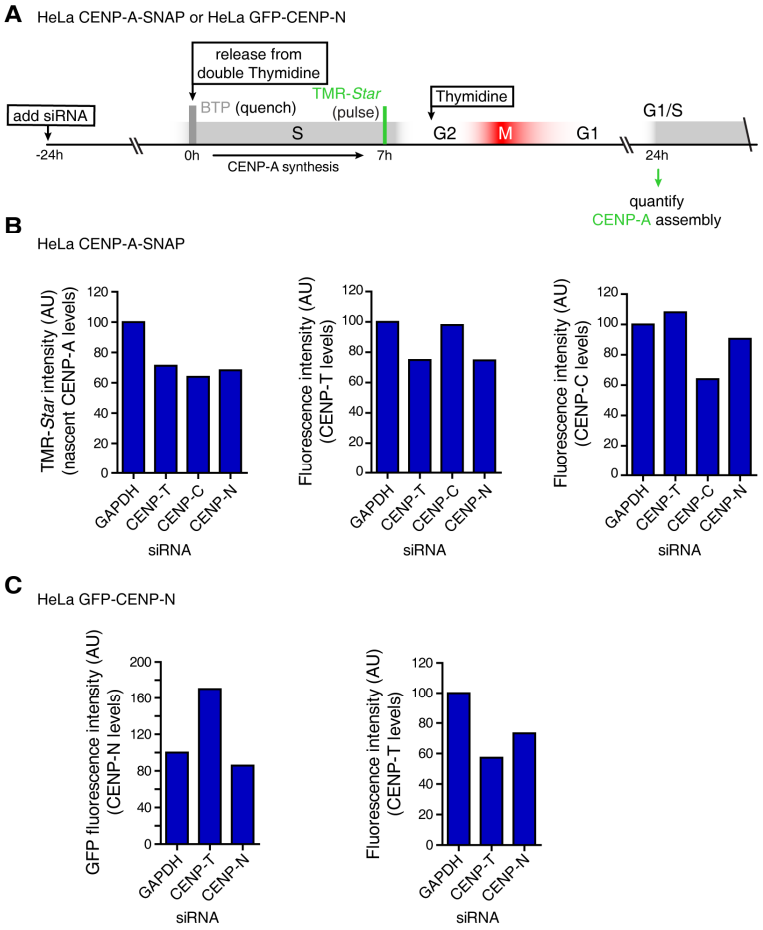

Figure 2.4. CENP-C, CENP-T and CENP-N are required for CENP-A assembly. (A) HeLa cells expressing CENP-A-SNAP were transfected with siRNAs against indicated targets (GAPDH serve as negative control), and synchronized by double Thymidine block combined with SNAP quench-chase-pulse labeling. Cells were cycled into the next cell cycle and collected at the next G1/S boundary (t = 24 hours). (B) Cells were imaged and counterstained for CENP-T (middle graph) and CENP-C (right graph). The levels of these proteins and CENP-A-TMR-*Star* intensity (left graph) were quantified in more than 6000 centromeres per condition. The graphs represent the fluorescence intensity of CENP-A-TMR-*Star* (left), CENP-T (middle) and CENP-C (right) in cells depleted of the indicated proteins. (C) HeLa cells expressing GFP-CENP-N were transfected with siRNAs and synchronized as in A. GFP-CENP-N (left) and CENP-T (right) levels were quantified in more than 3000 centromere per condition. Results from a single experiment are shown in B and C.

The majority of CCAN components are dependent on each other making it difficult to determine which components, if any, are directly involved in CENP-A assembly [Figure 2.4 B and C, and (Foltz et al., 2006; Liu et al., 2006; Okada et al., 2006; McClelland et al., 2007; Hori et al., 2008; Carroll et al., 2009, 2010). We decided to further analyse the role of CENP-N in CENP-A assembly because, concurrently with our results, our collaborators, Aaron Straight and Christopher Carroll at Stanford University School of Medicine identified CENP-N as the first protein to bind directly and specifically to CENP-A nucleosomes (Appendix 2, Carroll et al., 2009). We showed that depletion of CENP-N to ~50% of unperturbed levels is sufficient to cause a significant defect in CENP-A assembly (Figure 2.5 A-D). Additionally, we observed that CENP-N depletion causes a reduction of CENP-T levels at centromeres (Figure 2.4 B - middle graph, and C - right graph). Down regulation of CENP-N also causes a reduction on CENP-C levels, and has a more severe impact on CENP-H, CENP-I and CENP-K levels at centromeres (Appendix 2, Carroll et al., 2009).

Carroll et al., (2009) also showed that CENP-N binds CENP-A nucleosomes *in vitro*, independently of the underlying DNA sequence. CENP-N does not bind to H3 nucleosomes or prenucleosomal CENP-A/H4 complexes, but can bind nucleosomes containing a chimeric histone H3, in which a portion of H3 is replaced with the corresponding domain of CENP-A [named the CENP-A targeting domain (CATD)] that is sufficient to drive $H3^{CATD}$ to the centromere. Thus, CENP-N recognizes the structural information unique to CENP-A nucleosomes, which is determined by the CATD domain. Point mutations within the N terminus of CENP-N (R11A and R196A) impaired its binding to CENP-A nucleosomes but only slightly affect its centromeric localization. The conserved C terminus of CENP-N is in turn required for its binding to CENP-L, which connects CENP-N to the other CCAN components. Although CENP-N mutants that lack the C terminus can still bind efficiently CENP-A nucleosomes *in vitro*, they fail to bind CENP-A, CENP-H, CENP-K and CENP-L *in vivo*. Moreover, this mutant cannot localize to centromeres, suggesting that CENP-N association with other

CCAN components is important for its stabilization and recruitment to the centromere (Appendix 2, Carroll et al., 2009).

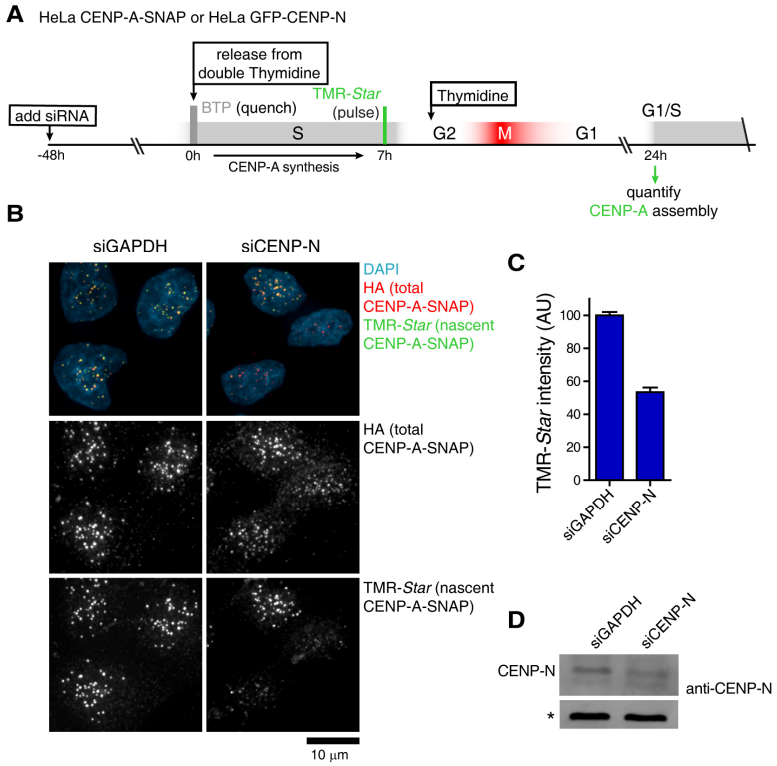

Figure 2.5. Depletion of CENP-N affects CENP-A assembly. (A) HeLa cells expressing CENP-A-SNAP were transfected with siRNA against CENP-N (GAPDH serves as negative control) and synchronized by double Thymidine block combined with SNAP quench-chase-pulse labeling. Cells were cycled into the next cell cycle and collected at the next G1/S boundary (t = 24 hours). (B) Cells were imaged and counterstained using an anti-HA antibody to distinguish steady state (CENP-A-SNAP-HA) from new (CENP-A-SNAP-TMR-*Star*) CENP-A. (C) The levels of CENP-A-TMR-*Star* intensity were quantified in more than 200 centromeres from 20 cells in each experiment. Mean and SEM of 3 replicates of each condition are shown. (D) HeLa cells expressing CENP-A-SNAP were transfected with siRNAs and synchronized as in A, followed by processing for SDS-PAGE and immunoblotting. Efficiency of depletion is assessed by CENP-N protein levels using anti-CENP-N antibody. Asterisk represents a cross reacting band that served as loading control.

Several CCAN components appear to be required for CENP-A assembly, but is unclear what is the role of these structural components in this pathway. These proteins are not likely to function as assembly factors since they are associated with the centromeric CENP-A fraction but not with the soluble fraction (Foltz et al., 2006, 2009; Dunleavy et al., 2009; Shuaib et al., 2010). Instead, they may form a recognition platform for specific CENP-A assembly factors or they can function as stabilizing factors once CENP-A is assembled.

Discussion and Conclusions

In this chapter we combined the use of SNAP labeling technology with siRNA mediated protein depletion, thereby enabling the identification of components involved in CENP-A assembly. We used two previously described CENP-A assembly factors, Mis18α and Mis18BP1^{HsKNL2}, to test the applicability of this novel approach. We confirmed that the Mis18 complex is targeted to centromeres prior to CENP-A and is required for the establishment of centromeric chromatin (Figures 2.1, 2.2 and 2.3). Contemporaneously, this approach was used to demonstrate that the CENP-A chaperone, HJURP is required for deposition of newly synthesised CENP-A (Foltz et al., 2009). Additionally, we showed that structural components of the centromere, CENP-C, CENP-T and CENP-N are also required for CENP-A assembly (Figures 2.4, 2.5, Appendix 2 and Carroll et al., 2009). Thus, we have demonstrated that the establishment of centromeric chromatin requires different classes of proteins, which likely have distinct roles in this process.

Being at right place at the right time: the Mis18 complex licenses centromeric chromatin for CENP-A assembly

The Mis18 complex arrives to the centromere during anaphase, preceding the assembly of nascent CENP-A (Figure 2.2). This localization pattern is strongly correlated with its role in CENP-A assembly. Indeed, depletion of any component of the Mis18 complex impairs the centromere targeting of the other components of the complex and causes a striking defect in the recruitment of nascent CENP-A to centromeric chromatin [Figure 2.1 and (Fujita et al., 2007; Maddox et al., 2007)]. Additionally, mutations in any of four conserved Cysteine residues of Mis18α abolish its centromeric localization and consequently impair CENP-A assembly (Fujita et al., 2007). This observation indicates that centromeric localization of the Mis18 complex is required for CENP-A targeting to

the centromeres. Although this complex is present at the right place at the right time and affects nascent CENP-A levels at the centromere, a direct interaction between CENP-A and the members of Mis18 complex has not been reported (Hayashi et al., 2004; Fujita et al., 2007; Lagana et al., 2010). Moreover, none of these proteins were found in association with either CENP-A nucleosomes or with prenucleosomal complexes (Foltz et al., 2006, 2009; Dunleavy et al., 2009; Shuaib et al., 2010). Therefore, the specific role of the Mis18 complex in CENP-A assembly is largely undefined. The localization pattern of this complex suggests that it is not directly involved in deposition of CENP-A but rather "licenses" or "primes" chromatin for CENP-A assembly.

It has been proposed that the role of the Mis18 complex might be related with histone acetylation, as addition of Trichostatin A (TSA), an inhibitor of histone deacetylases (HDACs), partially rescues CENP-A assembly in Mis18α depleted cells (Fujita et al., 2007). In addition, the Mis18 complex interacts with RbAp48 and RbAp46 (Fujita et al., 2007; Lagana et al., 2010), of which RbAp46 binds to histone H4 and also to Hat1, a histone acetyl transferase (Verreault et al., 1998). Based on this it was hypothesized that the Mis18 complex licenses the chromatin into a "competent" state for CENP-A assembly through histone acetylation (Fujita et al., 2007). However, several observations challenge this view. First, Hat1 is a generic acetyltransferase that acetylates all de novo synthesized H4 (Verreault et al., 1998), arguing against a specific role at the centromere. Second, TSA treatment leads to strong mitotic arrest, which prevents cells from reaching the cell cycle stage during which CENP-A is assembled and thus creating potential artifacts in measurements of CENP-A assembly (Ma et al., 2008). Third, transient TSA treatment leads to defective centromere function and, consequently, to chromosome segregation problems (Ekwall et al., 1997), inconsistent with a positive role in centromere assembly. Fourth, in fission yeast, centromeric chromatin is highly deacetylated and depletion of Mis18 and Mis16 results in a hyperacetylated centromeric chromatin domain, which contrasts with the findings in human cells (Hayashi et al., 2004). However, in Mis16 and Mis18

mutants, CENP-A^{Cnp1} is lost from the centromere and the region is invaded by canonical acetylated nucleosomes. Therefore the effect of Mis16 and Mis18 on yeast centromere acetylation may be indirect. Finally, in mammalian cells, centromeric chromatin is also hypoacetylated, but in a manner that is constitutive throughout the cell cycle (Ouspenski et al., 2003), which argues against a model where histone H4 acetylation plays an important role in CENP-A assembly. Alternatively, the Mis18 complex might have a role in modifying other aspects of centromeric chromatin structure, which may include a role in excision of canonical histones during G1 phase prior to or during CENP-A assembly. Another possibility is that, although the Mis18 complex does not bind CENP-A directly, it may be involved in recruiting a specific CENP-A assembly factor. Consistent with this last hypothesis, in *S. pombe*, Mis18 is required for the localization of Scm3, the fission yeast orthologue of the human CENP-A specific chaperone HJURP (Pidoux et al., 2009; Williams et al., 2009).

CCAN forms an epigenetic loop responsible for propagating the centromeric chromatin

Several CCAN components have been implicated in the assembly of CENP-A chromatin, but the specific role of these structural components in this pathway remains unclear. A role in CENP-A assembly is somewhat paradoxical, because the CCAN components are themselves dependent on CENP-A. The first CCAN components implicated in CENP-A assembly were the members of the CENP-H/I/K complex. The CENP-H/I/K complex is required for the centromere localization of the chromatin modulators FACT and CHD1, which are required for CENP-A assembly (Okada et al., 2009). FACT and CHD1 are localized at centromeres throughout the cell cycle in chicken DT40 cells (Okada et al., 2009) and were found to copurify with CENP-A chromatin in human cells (Obuse et al., 2004; Foltz et al., 2006; Izuta et al., 2006). However, they were not found in CENP-A prenucleosomal complexes, suggesting that they are not directly

involved in delivering CENP-A to the centromere (Dunleavy et al., 2009; Foltz et al., 2009; Okada et al., 2009; Shuaib et al., 2010). In flies, FACT and CHD1 interact with HIRA and have a role in replacing histone H3 by H3.3 (Konev et al., 2007; Nakayama et al., 2007). Thus, it is possible that these chromatin remodeling factors change the structure of centromeric chromatin to facilitate the replacement of canonical histone H3 with the histone H3 variant CENP-A, similar to their role in H3.3 assembly. Recently, it was proposed that histone H3.3 is deposited at centromeres during S phase as a placeholder for nascent CENP-A, that assembles in G1 phase (Dunleavy et al., 2011). Thus, an alternative possibility is that FACT and CDH1 are involved in this H3.3 deposition during S phase, which could influence the subsequent incorporation of CENP-A into centromeric nucleosomes.

Here, we have shown that CENP-C, CENP-T and CENP-N are also required for propagation of centromeric chromatin. Depletion of these proteins also leads to loss of the CENP-H/I/K complex from the centromeres (Hori et al., 2008; Carroll et al., 2009, 2010). The defect we observed could therefore be indirect and caused by the loss of this complex. However, since CENP-C and CENP-N bind directly to CENP-A nucleosomes, it is possible that they have a more direct role in CENP-A assembly (Carroll et al., 2009, 2010). Importantly, all these proteins depend on CENP-A for their centromeric localization. In this way, by promoting CENP-A assembly, these factors generate more of their own binding sites which in turn expands the platform for CENP-A assembly. As such, CENP-N, CENP-C and potentially other CCAN components form an epigenetic positive feedback loop, in part responsible for propagation of active centromeres in mammalian cells. One likely possibility is that CCAN components recruit CENP-A specific assembly factors. Indeed, two recent studies have demonstrated that CENP-C directly interacts with Mis18BP1 in *X. laevis* egg extracts, *X. laevis* S3 cells and mouse ES cells (Moree et al., 2011; Dambacher et al., 2012). In *X. laevis*, two isoforms of Mis18BP1 were identified, one that is targeted to centromeres in metaphase and other that is targeted only in

interphase, both of which are required for CENP-A assembly (Moree et al., 2011). In this system, CENP-C is required for centromere targeting of the Mis18BP1 during metaphase but not during interphase (Moree et al., 2011), suggesting the existence of a second mechanism that recruits Mis18BP1 to *X. laevis* centromeres. In human cells, an interaction between Mis18BP1^{HsKNL2} and CENP-C has not been observed (Fujita et al., 2007; Lagana et al., 2010). However, it remains possible that these proteins only interact during a short cell cycle window which has escaped detection by biochemical means.

Overexpression of CENP-A leads to general misincorporation throughout chromatin in both human and fly cells (Van Hooser et al., 2001; Heun et al., 2006; Gascoigne et al., 2011). Strong CENP-A^{CID} overexpression in flies leads to the formation of functional, ectopic centromeres/kinetochores at distinct chromatin loci. However, in human cells, overexpression and misincorporation of CENP-A results in the corecruitment of only two proteins, CENP-N and Mis18α, out of the 16 centromere/kinetochore components tested. In addition, CENP-C could also be driven to the general chromatin, but only in conditions where it was co-overexpressed with CENP-A (Gascoigne et al., 2011). These results suggest that, in mammalian cells, Mis18α might be recruited to centromeres by CENP-N. Alternatively, the Mis18 complex may recognize a specific structural signature formed by CENP-A chromatin.

In this chapter, we also showed that CENP-N depletion reduces the levels of CENP-T at the centromere (Figure 2.4 B and C). The decrease of CENP-T levels was similar to the one observed for CENP-C, but not as dramatic as the decrease observed in CENP-H, CENP-I and CENP-K levels at centromeres (Appendix 2, Carroll et al., 2009). This could be explained by the fact that the CENP-T/W complex and CENP-C make direct contacts with centromeric chromatin, and like CENP-N, assemble into centromeres upstream of the other CCAN components (Hori et al., 2008; Carroll et al., 2009, 2010; Gascoigne et al., 2011; Guse et al., 2011; Nishino et al., 2012). CENP-N, CENP-T and CENP-C might be independently targeted to the centromere and cooperate in decoding

the epigenetic information carried by CENP-A nucleosomes and in forming a critical platform to recruit the downstream centromere/kinetochore proteins (Hori et al., 2008; Carroll et al., 2009, 2010; Gascoigne et al., 2011; Guse et al., 2011; Przewloka et al., 2011; Screpanti et al., 2011). Consistent with this idea, in the absence of CENP-A, the N-terminal regions of CENP-C and CENP-T, when tethered to naive chromatin at high density, are sufficient to direct the assembly of the kinetochore during mitosis. Interestingly, these ectopic CENP-T/CENP-C foci do not recruit Mis18α or CENP-A, while the entire downstream centromere/kinetochore complex appears to be present (Gascoigne et al., 2011).

In conclusion, the CCAN is not only required for kinetochore assembly during mitosis, but it also plays a role in the establishment and maintenance of centromeric chromatin. Different CCAN components may have a differential role in CENP-A assembly. However, a direct involvement is difficult to test, because many CCAN proteins depend on each other for their centromere localization (Foltz et al., 2006; Liu et al., 2006; Okada et al., 2006; McClelland et al., 2007; Hori et al., 2008; Carroll et al., 2009, 2010). Interestingly, some CCAN components appear to negatively regulate the localization of upstream components, like CENP-N, preventing their over-accumulation (Figure 2.4 C - left graph and McClelland et al., 2007). Thus, both positive and negative regulation among CCAN proteins might be important for the establishment and maintenance of this protein complex.

CENP-A assembly occurs in three distinct steps: licensing, assembly and stabilization

The question of how CENP-A loading is initiated and restricted to the centromeric region has been intensively explored in the last few years. A number of proteins that affect CENP-A localization at centromeres have been identified, in different organisms and using different approaches (Table 2.2). Collectively, these findings have led to a model suggesting that the process of CENP-A assembly may occur in three steps: licensing, assembly and stabilization/maturation (Figure 2.6).

In this model, the licensing step is performed by the Mis18 complex, which includes Mis18α, Mis18β and Mis18BP1^{HsKNL2} (Figure 2.6 A). Centromere targeting of the Mis18 complex precedes CENP-A assembly. In addition, while CENP-A assembly continues throughout G1 (Lagana et al., 2010), the Mis18 complex dissociates from the centromere in mid G1 phase, before CENP-A assembly is completed (Figure 2.3 and Fujita et al., 2007). This indicates that the Mis18 complex is only involved in the initial step of CENP-A assembly. So far, a molecular link between CENP-A and the Mis18 complex has not been found (Foltz et al., 2006, 2009; Fujita et al., 2007; Dunleavy et al., 2009; Lagana et al., 2010). Intriguingly, the targeting of Mis18 complex to centromeres is largely unaffected by severe CENP-A depletions (Hayashi et al., 2004; Fujita et al., 2007). However, it cannot be excluded that residual CENP-A levels are sufficient to recruit the Mis18 complex. Indeed, chicken cells in which CENP-A is conditionally knocked out can proliferate normally for up to 4 days after the *CENP-A* gene is silenced (Régnier et al., 2005). Similarly, in HeLa cells only 10% of the initial levels of CENP-A are sufficient to support the assembly of a normal kinetochore and promote accurate cell division (Liu et al., 2006). Further efforts will be required to understand how Mis18 is targeted to centromeres and to indentify the molecular basis of this licensing step. As discussed above, two

possible candidates for the recruitment step of the Mis18 complex are CENP-C and CENP-N.

The major player in the assembly step is the human Holliday junction recognizing protein (HJURP) in human cells or its fission yeast homologue Scm3 (Kato et al., 2007; Dunleavy et al., 2009; Foltz et al., 2009; Pidoux et al., 2009; Sanchez-Pulido et al., 2009; Williams et al., 2009; Shuaib et al., 2010). Scm3 is able to bind CENP-A^{Cnp1}, both *in vivo* and *in vitro*, and is required for CENP-A^{Cnp1} recruitment to fission yeast centromeres (Pidoux et al., 2009; Williams et al., 2009). HJURP was found in CENP-A prenucleosomal complexes together with histone H4, Npm1 and RbAp48 (Dunleavy et al., 2009; Foltz et al., 2009; Shuaib et al., 2010). Npm1 appears to be present in prenucleosomal complexes distinct from those containing HJURP, which suggests that, perhaps together with RbAp48, it may complex with an inactive pool of nascent CENP-A and transfer CENP-A/H4 to HJURP (Figure 2.6 B). HJURP, as CENP-A itself, is localized to the centromeres in late telophase/early G1 and is required for CENP-A assembly [Figure 2.1 B, C and (Jansen et al., 2007; Dunleavy et al., 2009; Foltz et al., 2009)]. These observations suggested that HJURP and Scm3 might function as specific chaperones that deliver CENP-A into human and fission yeast centromeres, respectively (Figure 2.6 B). Confirming this hypothesis, a recent study has shown that HJURP has CENP-A chromatin assembly activity *in vitro* (Barnhart et al., 2011). To fully understand this CENP-A assembly step it will be important to demonstrate how HJURP/CENP-A prenucleosomal complex is targeted to the centromere. In fission yeast, Scm3 interacts with Mis18 *in vitro* and its localization depends on Mis18 and on the constitutive centromere components CENP-K^{Sim4} and CENP-I^{Mis6} (Pidoux et al., 2009; Williams et al., 2009). Recently, it was shown that the Mis18 complex is also required for HJURP recruitment to human centromeres (Barnhart et al., 2011). Interestingly, HJURP targeting to an ectopic chromosomal location using the LacI/LacO system is sufficient to drive stable recruitment of CENP-A, independently of the Mis18 complex (Barnhart et al., 2011). These results strongly suggest that the role of

the Mis18 complex in CENP-A assembly is to recruit HJURP to the centromere, which in turn assembles CENP-A into centromeric chromatin. However, how the Mis18 complex recruits HJURP to the centromere remains unclear. In human cells, no interaction is reported between HJURP and the Mis18 complex. The link between them may be mediated by RbAp46/48 that interacts with both HJURP and the Mis18 complex (Fujita et al., 2007; Dunleavy et al., 2009; Lagana et al., 2010; Shuaib et al., 2010). In support of this, in fission yeast, Scm3 and Mis18 are also capable of binding Mis16, the fission yeast homologue of RbAp46/48 (Hayashi et al., 2004; Williams et al., 2009).

More recently, new players have been identified that localize to the centromere during mid and late G1 phase and affect CENP-A levels. This adds a new dimension to the process of CENP-A assembly and led to the proposal that the establishment of CENP-A chromatin includes a stabilization/maturation step following initial deposition (Figure 2.6 C). The, as of yet, poorly defined role of stabilizing newly incorporated CENP-A may be executed by Rsf-1, a member of the ATP-dependent chromatin remodeling and spacing factor (RSF) complex, and by MgcRacGAP (Perpelescu et al., 2009; Lagana et al., 2010). Both were initially identified as members of the Interphase Centromere Complex (ICEN) (Izuta et al., 2006), but were shown to localize to centromeres during a short time window, from mid to late G1 in the more recent reports.

Rsf-1 depletion does not affect centromeric levels of CENP-A per se but renders CENP-A sensitive to salt extraction suggesting CENP-A chromatin is unstable in the absence of Rsf-1 (Perpelescu et al., 2009). How the RSF complex is targeted to centromeres remains unknown, but the fact that it copurified with CENP-A chromatin as an ICEN component suggests a close interaction with either CENP-A nucleosomes or another CCAN component (Obuse et al., 2004; Izuta et al., 2006). Interestingly, in fission yeast a similar ATP-dependent chromatin remodeling factor, Hrp1 has been shown to associate with centromeric repeats and affect centromeric CENP-A^{Cnp1} levels (Walfridsson et al., 2005).

MgcRacGAP was found in Mis18BP1^{HsKNL2} affinity purifications (Lagana et al., 2010), as well as in CENP-A pulldown experiments (Obuse et al., 2004; Izuta et al., 2006). However, the timing of centromere localization differs for these two proteins. It was shown that MgcRacGAP [together with Ect2, a Rho family guanine nucleotide exchange factor (GEF), and the small GTPases Cdc42 and Rac], is specifically required for centromere localization of newly synthesized CENP-A. Whether MgcRacGAP is involved in the initial assembly step in early G1 phase or in a later step has not been directly addressed. However, its conspicuous centromeric localization in late G1, when CENP-A assembly is nearly completed, suggests it may be part of a “quality control” mechanism that stabilizes centromeric chromatin before S phase entry (Lagana et al., 2010).

The RSF complex and MgcRacGAP may act together during mid to late G1 phase to stabilize the newly assembled CENP-A nucleosomes allowing the maintenance of these nucleosomes throughout the cell cycle. This stabilization action at centromeric chromatin might include proper spacing between CENP-A nucleosomes or represent a step in which the nucleosome reach full octameric occupancy with the addition of histones H2A and H2B (Black and Cleveland, 2011), possibly resulting in increased resistance to salt. Further studies are required to dissect the molecular mechanism underlying this stabilization or maturation step of CENP-A assembly.

Together these observations imply a model in which centromeric chromatin is primed by the Mis18 complex that enables the recruitment of the HJURP/CENP-A/H4 prenucleosomal complex to the centromere resulting in nucleosome assembly. Finally centromeric chromatin reaches a mature, stable form through the action of the RSF complex and McgRacGAP (Figure 2.6).

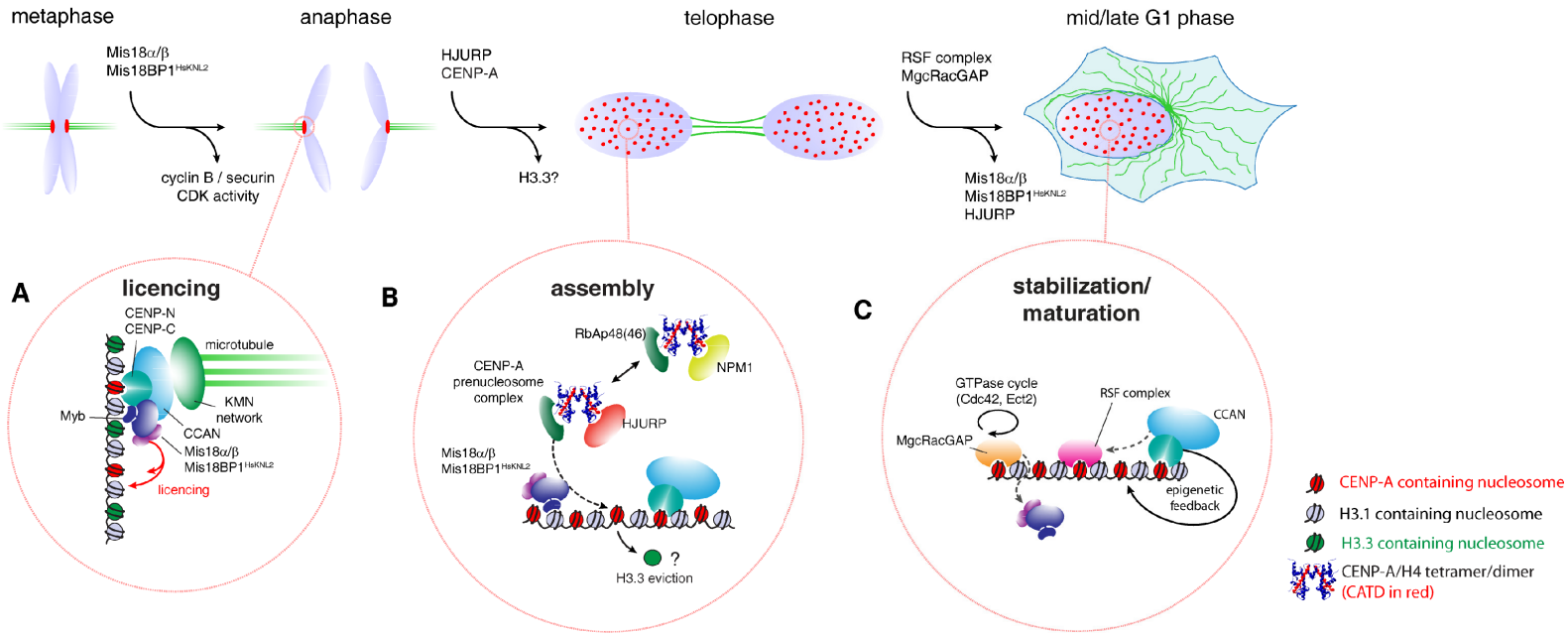

Figure 2.6. Model of CENP-A assembly process in human cells. CENP-A assembly is proposed to occur in three steps: licensing, assembly and stabilization/maturation. (A) The licensing step occurs upon mitotic exit, during anaphase, rendering centromeric chromatin competent for new CENP-A incorporation. The main players during this step are Mis18α, Mis18β and Mis18BP1^{HsKNL2} (Mis18 complex). I propose that the Mis18 complex is targeted to the centromere through action of CENP-N and/or CENP-C. (B) Once centromeric chromatin is licensed, during late telophase/early G1, HJURP delivers CENP-A/H4 prenucleosomal complexes to the centromere. HJURP targeting requires the Mis18 complex, which recognizes the prenucleosomes possibly through an interaction with RbAp46/48. CENP-A assembly in this step possibly involves an exchange with histone H3.3 (Dunleavy et al., 2011). The newly assembled CENP-A nucleosomes remain in a premature/unstable state (represented in the figure as the red misaligned nucleosomes. C) This chromatin state is stabilized during mid to late G1 through a combined action of the RSF complex and a GTPase cycle that involves MgcRacGAp, Cdc42 and Ect2.

Table 2.2. Overview of proteins involved in CENP-A assembly in different organisms			
Protein	Organisms	Role in CENP-A assembly	References
CENP-H	*G. gallus*	*Stabilizes assembled CENP-A nucleosomes or recruits proteins actively involved in CENP-A assembly*	(Okada et al., 2006)
CENP-I^{SpMis6}	*G. gallus*, *S. pombe*		(Takahashi et al., 2000; Okada et al., 2006)
CENP-K^{SpSim4}	*G. gallus*, *S. pombe*		(Pidoux et al., 2003; Okada et al., 2006)
CENP-M^{SpMis17}	*G. gallus*, *S. pombe*		(Hayashi et al., 2004; Okada et al., 2006; Perpelescu and Fukagawa, 2011)
CENP-T	*H. sapiens*		This thesis
Mis12	*H. sapiens*		(Kline et al., 2006)
CENP-N^{SpMis15}	*H. sapiens*, *S. pombe*	*Recruits Mis18 complex to the centromere and/or stabilizes assembled CENP-A nucleosomes*	This thesis and (Gascoigne et al., 2011)
CENP-C	*H. sapiens, D. melanogaster*		This thesis and (Erhardt et al., 2008)
Mis18α^{SpMis18}	*H. sapiens*, *S. pombe*	Licenses centromeric chromatin for CENP-A assembly	(Hayashi et al., 2004; Fujita et al., 2007)
Mis18β ^{SpMis18}	*H. sapiens*, *S. pombe*		
Mis18BP1^{CeKNL2}	*H. sapiens*, *M. musculus*, *X. laevis*, *C. elegans*		(Fujita et al., 2007; Maddox et al., 2007; Moree et al., 2011; Dambacher et al., 2012)
RbAp46^{SpMis16}	*H. sapiens*, *S. pombe*	*Connects Mis18 complex and HJURP*	(Hayashi et al., 2004; Fujita et al., 2007)
RbAp48^{SpMis16}	*H. sapiens*, *S. pombe*		
HJURP^{SpScm3}	*H. sapiens*, *X. laevis*, *S. pombe*	Binds to prenucleosomal complexes of CENP-A and assembles them into centromeric chromatin	(Dunleavy et al., 2009; Foltz et al., 2009; Pidoux et al., 2009; Williams et al., 2009; Shuaib et al., 2010; Bernad et al., 2011)

Table 2.2. (continued)			
Protein	Organisms	Role in CENP-A assembly	References
Rsf-1	*H. sapiens*	*Stabilizes or matures centromeric chromatin after CENP-A assembly*	(Perpelescu et al., 2009)
MgcRacGap	*H. sapiens*		(Lagana et al., 2010)
Ect2	*H. sapiens*		
Cdc42	*H. sapiens*		
CHD1^{SpHrp1}	*H. sapiens*, *S. pombe*	Histone deposition and/or exchange	(Walfridsson et al., 2005; Okada et al., 2009)
FACT	*H. sapiens*		
Sim3	*S. pombe*	Upstream chaperone of CENP-A^{Cnp1}	(Dunleavy et al., 2007)
Ams2	*S. pombe*	Required for CENP-A^{Cnp1} assembly in S phase	(Chen et al., 2003; Takahashi et al., 2005)
Cal1	*D. melanogaster*	Licenses the centromere and binds to prenucleosomal CENP-A^{CID} directing its assembly.	(Erhardt et al., 2008)
Cyclin A	*D. melanogaster*	*Prevents premature destruction of a CENP-A^{CID} assembly factor*	
Rca1	*D. melanogaster*	*Prevents premature destruction of a CENP-A^{CID} assembly factor*	
Condensin II	*X. laevis*	Prevents CENP-A eviction	(Bernad et al., 2011)

Names of proteins listed in superscript correspond to homologues, and the organisms in which these homologues are present are indicated by the two letters before the name of the protein. Italic text represent roles in CENP-A assembly pathway proposed in this thesis, which need to be tested in future studies. Note that only organisms in which centromeres are epigenetically inherited are listed.

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Chapter 3 – Cell cycle control of CENP-A assembly is maintained by Cdk activity

Abstract

The centromere is a specialized chromosomal locus that drives the assembly of the kinetochore, thereby allowing proper chromosome segregation during mitosis. CENP-A, a histone H3 variant present exclusively at centromeres, is considered an epigenetic mark responsible for centromere function and propagation. Unlike canonical histones, which are assembled during DNA replication throughout chromatin, the assembly of nascent CENP-A into centromeric nucleosomes is restricted to early G1 phase of the cell cycle. Whilw several proteins have been shown to play a role in CENP-A loading into chromatin, the mechanism that is controlling the strict cell cycle dependent assembly of CENP-A remains unknown. Entry into G1 is dictated by loss of Cdk1 activity through APC/C-dependent degradation of its activator cyclin B. Using pharmacological and genetic experiments we have now found that this major regulator of mitosis is also controlling the timing of CENP-A assembly. Inhibition of Cdk1 and Cdk2 is sufficient to induce rapid CENP-A assembly without a strict need for passage through mitosis. We also demonstrated that neither *de novo* synthesis nor protein destruction is required to trigger CENP-A assembly upon inhibition of Cdk activity. Based on these results we propose a model in which the CENP-A assembly machinery is present throughout the cell cycle but is kept in an inactive, phosphorylated state by Cdk1 and Cdk2 activities.

Introduction

Accurate segregation of newly replicated chromosomes during mitosis is crucial to maintain genome integrity and to prevent aneuploidy, a major hallmark of human cancers (Weaver et al., 2007). Central to this process is the kinetochore which forms the chromosomal attachment site for spindle microtubules and is required for chromosome movement and mitotic checkpoint signaling (Cheeseman and Desai, 2008). The centromere is a unique constitutive chromatin domain that assembles the kinetochore during mitosis and is therefore essential for proper chromosome segregation and mitotic progression (Allshire and Karpen, 2008). Centromere specification and function is controlled epigenetically in most eukaryotes, as they lack a specific DNA sequence that is either sufficient or required to propagate the centromere (Vafa and Sullivan, 1997; Warburton et al., 1997; Warburton, 2004). The histone H3 variant Centromere Protein A (CENP-A) is present at centromeric chromatin in all eukaryotes and is necessary and sufficient to nucleate the entire centromere/kinetochore complex (Régnier et al., 2005; Foltz et al., 2006; Liu et al., 2006; Barnhart et al., 2011; Olszak et al., 2011). This has led to the proposal that CENP-A functions as an epigenetic mark that defines the centromere (Vafa and Sullivan, 1997; Warburton et al., 1997; Allshire and Karpen, 2008; Silva and Jansen, 2009). Consistent with this notion, it was recently demonstrated that the presence of CENP-A in the chromatin is sufficient to nucleate the formation of functional and heritable centromeres (Mendiburo et al., 2011). CENP-A containing nucleosomes are extremely stable and maintained throughout the cell cycle, being redistributed between the two sister chromatids during S phase (Jansen et al., 2007; Hemmerich et al., 2008; Dunleavy et al., 2011). Importantly, unlike the assembly of the canonical histone H3.1, CENP-A assembly into centromeric chromatin is uncoupled from DNA replication and, at least in metazoans, is restricted to late mitosis/early G1 phase of the cell cycle (Shelby et al., 2000; Jansen et al., 2007; Schuh et al., 2007; Hemmerich et al., 2008;

Bernad et al., 2011; Moree et al., 2011). Assembly of CENP-A strictly depends on passage through mitosis (Jansen et al., 2007; Schuh et al., 2007; Bernad et al., 2011; Moree et al., 2011), ensuring a direct coupling of centromere inheritance with cell cycle progression. Although several proteins have been identified to have a role in CENP-A deposition at centromeres (Chapter 2), the mitotic trigger that initiates and restricts centromere propagation to late mitosis/early G1 phase has not been identified. Possible candidates for this have been previously proposed, including changes in nuclear architecture (Jansen et al., 2007), anaphase promoting complex/cyclosome (APC/C)-mediated destruction of a specific inhibitor of CENP-A assembly (Erhardt et al., 2008), or assembly of a proper kinetochore-microtubule interface (Mellone and Allshire, 2003; Jansen et al., 2007; Allshire and Karpen, 2008).

Here, we determined the identity of the molecular trigger that temporally controls CENP-A assembly. We revealed that inhibiting cyclin-dependent kinase 1 (Cdk1) and Cdk2 in any phase of the cell cycle is sufficient to trigger rapid CENP-A assembly. Thus, our results point to a simple mechanism that excludes the need for any active involvement of mitosis in subsequent CENP-A assembly, other than the concomitant down regulation of Cdk activity upon mitotic exit.

Material and Methods

Cell lines and constructs

HeLa cells and their derivatives were cultured as described in Chapter 2. A CENP-N-SNAP-3xHA construct named pLJ363 was generated by inserting a PCR-generated fragment carrying the human CENP-N open reading frame flanked by KpnI and XhoI sites into corresponding sites of pSS26m (Covalys) containing an additional triple HA tag (3xHA) at its C-terminus. The resulting CENP-N-SNAP-3xHA (referred to as CENP-N-SNAP throughout this Chapter) was subcloned into a pBABE vector resulting in pLJ365. A HeLa cell line stably expressing CENP-N-SNAP was generated by Moloney murine leukemia retroviral delivery as previously described (Shah et al., 2004). Cells stably expressing CENP-N-SNAP were selected using 5 µg/mL Blasticidin S (Invitrogen) and single-cell sorted in a MoFlo High-Speed Cell Sorter (Beckman Coulter, USA). The resulting monoclonal lines were expanded and selected by fluorescence microscopy for expression level and localization.

DMEM-F12 (Gibco) medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-Glutamine, 100 U/mL Penicillin, 100 µg/mL Streptomycin and 3,48 g/L Sodium Bicarbonate (all from Gibco) was used to culture nontransformed telomerase-expressing human retinal pigment epithelial cells (hTERT-RPE) stably expressing CENP-A-SNAP at 37°C and 5%CO_2. This cell line was generated by retroviral delivery of a construct carrying CENP-A-SNAP-3xHA (Jansen et al., 2007) and selected using 10 µg/mL of Blasticidin S (Invitrogen) analogous to HeLa cell lines described above.

DT40 cell lines were cultured in RPMI1640 medium supplemented with 50 µM β-mercaptoethanol, 10% FBS and 1% chicken serum (all from Gibco) at 39°C 5%CO_2. A 3xHA-SNAP-CENP-A construct named pLJ404 was generated by inserting a PCR-generated fragment carrying the chicken CENP-A open reading frame (Gift from T. Fukagawa, NIG, Mishima, Japan) flanked by BamHI and XbaI

sites into corresponding sites of pSS26m (Covalys) containing an additional triple HA tag (3xHA) at its N-terminus. The resulting 3xHA-SNAP-CENP-A fusion protein (referred to as SNAP-CENP-A throughout this thesis) was subcloned into p3XFLAG-CMV-14 (Sigma) resulting in pLJ410 (including a STOP codon and excluding FLAG from the ORF). Stable lines expressing SNAP-CENP-A were created in DT40 *cdk1as* and *cdk1as/cdk2*$^{-/-}$ cells (Hochegger et al., 2007) by electroporation with a Gene Pulser apparatus (BioRad) at 550 V and 25 µF as previously described (Sonoda et al., 1998). *cdk1as* and *cdk1as/cdk2*$^{-/-}$ cell lines were selected using 0,5 µg/mL of Puromycin (Calbiochem) and 500 µg/mL Zeocin (Invivogen), respectively. Clonal cell lines expressing SNAP-CENP-A at subendogenous levels were selected by fluorescence microscopy after TMR-*Star* labeling (Figure 3.11 C and C') and by western-blot using anti-chicken CENP-A (Gift from T. Fukagawa, NIG, Mishima, Japan), and anti-HA (HA11, Covance Research Products, Inc.) antibodies (Figure 3.1).

Immunoblotting

Extracts of 10^5 (HeLa) or 2 x 10^6 (DT40) cells were separated in a 12% (HeLa) or 15% (DT40) SDS-PAGE gel and transferred to a PVDF membrane. In the case of HeLa cells, blots were probed with anti-cyclin B1 (sc-245, Santa Cruz) at 1:500 dilution, anti-Actin (A2066, Sigma) at 1:1000 dilution and anti-IκB-α (sc-371, Santa Cruz) at 1:1000 dilution. To screen DT40 monoclonal lines stably expressing subendogenous levels of 3xHA-SNAP-CENP-A we used anti-chicken-CENP-A (gift from T. Fukagawa, NIG, Mishima, Japan) and anti-HA (HA11, Covance Research Products, Inc.) antibodies at dilutions of 1:3000 and 1:1000, respectively. Anti-mouse and anti-rabbit HRP-conjugated secondary antibodies were purchased from Jackson Immunoresearch Laboratories.

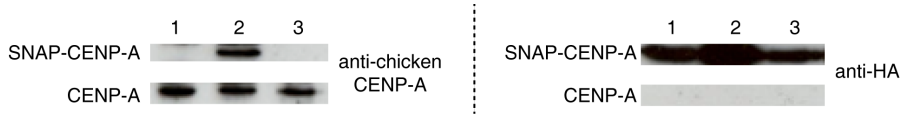

Figure 3.1. DT40 cell lines stably expressing low levels of SNAP-CENP-A. Western blots are shown for 3 selected clonal lines probed using anti-chicken-CENP-A, which recognize both endogenous and SNAP-tagged CENP-A, and anti-HA, which recognize only SNAP-tagged CENP-A. We picked DT40 *cdk1as* (1) and *cdk1as/cdk2*$^{-/-}$ (3) cell lines expressing SNAP-CENP-A at subendogenous levels (below detection using anti-CENP-A). A cell line expressing SNAP-CENP-A at nearly endogenous levels (2) was used as a positive control to demonstrate that the anti-chicken-CENP-A antibody is able to recognize SNAP-tagged CENP-A.

Cell synchronization and drug treatments

The different drugs and respective concentrations used in human cells are listed below. Roscovitine, Purvalanol A (both from Sigma) and the specific Cdk1 inhibitor RO3306 (Calbiochem) were used at 100 µM, 25 µM and 90 µM, respectively. MG132 and Cyclohexamide (both from Sigma) were used at 24 µM and 10 µg/mL, respectively. DMSO (Sigma) was used in control conditions. Colcemid (Gibco) and Nocodazole (Sigma) were used at 1 µg/mL and 100 ng/mL, respectively. TNFα (R&D Systems) was used at 50 ng/ml (Seldon et al., 2007). HeLa cells were synchronized as described in Chapter 2.

DT40 cells were treated with 1 µM or 10 µM of 1NM-PP1 (synthesized by Chris Larch and Hansjoerg Streicher, Sussex University, UK) for partial or strong inhibition of Cdk1as activity, respectively. DT40 cells were synchronized with 100 ng/mL Nocodazole or 2 mM Hydroxyurea (HU) (both from Sigma). Following release from an HU induced S phase arrest (Figure 3.16), DT40 cells were allowed to enter S phase for 30 minutes prior to 1NM-PP1 addition to avoid rearrest due to Cdk1 requirement to enter S phase.

SNAP quench-chase-pulse labeling

HeLa cells expressing CENP-N-SNAP or CENP-A-SNAP were pulse labeled by addition of 2 µM BTP (SNAP-Cell Block; New England Biolabs), in growth medium for 30 minutes at 37°C and 5% CO_2 for irreversible, nonfluorescent labeling of the preexisting CENP-N-SNAP or CENP-A-SNAP pool, respectively. We refer to this step as "quench". Following quench, cells were chased for 7 hours to allow synthesis of new and unlabeled CENP-N-SNAP or CENP-A-SNAP pools. This newly synthesized pool was labeled with 2 µM TMR-*Star* (SNAP-Cell TMR-*Star*; New England Biolabs), in growth medium for 15 minutes at 37°C, 5% CO_2, thereby fluorescently labeling the nascent CENP-N-SNAP or CENP-A-SNAP pools, specifically. hTERT-RPE cells stably expressing CENP-A-SNAP were quenched and pulse labeled as described for HeLa cells. DT40 cells were quenched as described for HeLa cells followed by a chase time of 3 hours and 30 minutes at 39 °C and 5% CO_2. Cells were pulse labeled with 5 µM of TMR-*Star*. After each labeling step (fluorescent and nonfluorescent) cells were washed twice with medium and reincubated at the appropriate temperature to allow excess SNAP substrate to be released from cells. After 30 minutes, cells were washed again once in medium.

Immunofluorescence

HeLa or hTERT-RPE cells were grown on glass coverslips [thickness: 1.5, coated with poly-L-Lysine (Sigma)] and fixed with 4% formaldehyde for 10 minutes. DT40 cells were resuspended in PBS at a concentration of 2 x 10^5 cells/mL and cytospun at 800 rpm during 5 min. Cells were then fixed with 4% formaldehyde for 10 minutes. For BrdU staining (Figure 3.8), HeLa cells were fixed in Methanol/Acetone (1:1; Sigma) for 3 minutes, acid treated with 2 M HCl during 10 min at RT followed by 3 x 10 minutes washes with 100 mM Borax (Sigma). HeLa cells were extracted with 0,1% Triton X-100 and stained with anti-cyclin B1 (sc-245, Santa Cruz) at 1:50 dilution, anti-CENP-T [gift from D. Foltz,

University of Virginia, Charlottesville, VA; (Barnhart et al., 2011)] at 1:1000 dilution, anti-CENP-C (gift from D. Foltz, University of Virginia, Charlottesville, VA) at 1:10000 dilution, anti-α-tubulin (clone YL1/2, Serotec) at 1:2500 dilution and anti-BrdU (MoBU-1, Santa Cruz) at 1:100 dilution. hTERT-RPE cells were extracted with 0,1% Triton X-100 and stained with anti-α-tubulin at 1:2500 and anti-HA (clone HA11, Covance) at the concentration of 1 µg/mL. DT40 cells were extracted with 0,1% Triton X-100 and stained with anti-chicken CENP-O (gift from T. Fukagawa, National Institute of Genetics, Mishima, Japan) at 1:3000 dilution, and anti-chicken cyclin B2 (gift from E. Nigg, University of Basel, Basel, Switzerland) at 1:50 dilution. Secondary antibodies (FITC- or Cy5-conjugated anti-mouse, FITC- or Cy5-conjugated anti-rabbit and FITC-conjugated anti-rat) were obtained from Jackson Immunoresearch Laboratories. Dy680 conjugated anti-mouse antibodies were from Rockland Immunochemicals. Cells were stained with DAPI (4',6-diamidino-2-phenylindole; Sigma) before mounting in Mowiol.

Microscopy

Widefield fluorescence microscopy was performed using a DeltaVision Core system (Applied Precision) as described in Chapter 2. Quantified images were acquired using the same exposure conditions for each fluorescent channel. TRITC, FITC and Cy5 images of a uniformly slide were automatically flatfield- and camera-noise-corrected using softWoRx (Applied Precision). All images presented are maximum intensity projections of deconvolved pictures, except for figure 3.2, in which the pictures are not deconvolved.

Fluorescence Quantification

Centromeric TMR-*Star* fluorescence intensity was quantified using *CRaQ* (Bodor et al., 2012). Standard parameter settings for *CRaQ* were optimized as follows: square size = 7 (Figure 3.4 E) or 5 (Figure 3.13 C); minimum circularity = 0.95; maximum feret's diameter = 7; minimum centromere size = 4; maximum centromere size = 35; threshold factor = 1.1; chromatic aberration correction = (0,0).

Flow cytometry

DT40 cells (10^6) were harvested and fixed during 1 hour at 4°C with 70% ethanol. Cells were washed twice in PBS containing 3% BSA (Sigma) and incubated for 3 hours at RT with 5 µg/ml propidium iodide (PI; Sigma) and 200 µg/mL of RNaseA in PBS containing 3% BSA. Subsequently flow-cytometric analysis was performed on a FACScan (Becton Dickinson) or FACS Canto (Becton Dickinson) using CellQuest and FACSDiva software, respectively.

For BrdU staining we used an anti-BrdU antibody (347580, Becton and Dickison). Cells were fixed as described above and acid treated with 2 M HCl for 30 minutes. Cells were washed twice with 100 mM Borax, followed by extraction 0,5% Tween 20. Cells were incubated with 10 µL of anti-BrdU antibody for 30 minutes and subsequently stained with a Cy5 secondary antibody (Jackson Immunoresearch) for additional 30 minutes and with PI as described above. Cells were analyzed on a CyAn ADP (Beckman Coulter).

Results

CENP-N loading at centromeres is not restricted to G1 phase of the cell cycle

Assembly of CENP-A nucleosomes is tightly restricted to G1 phase of the cell cycle (Jansen et al., 2007; Schuh et al., 2007; Hemmerich et al., 2008). Subsequently, CENP-N was the first protein to be shown to bind directly and specifically to CENP-A nucleosomes *in vitro* (Carroll et al., 2009). This led us to hypothesize that CENP-N may have a turnover rate and timing of assembly similar to CENP-A. Indeed, levels of CENP-N at the centromere decrease dramatically at metaphase and increase as cell exit mitosis (McClelland et al., 2007), coinciding with the time when CENP-A assembles into centromeric chromatin. This suggests a possible cell cycle dependent control of centromeric CENP-N levels. To further analyze the dynamics of CENP-N at the centromeres across the cell cycle we used SNAP-based fluorescent quench-chase-pulse labeling, previously described in Chapter 2. We first quenched the existing CENP-N-SNAP pool with a nonfluorescent substrate (BTP) in an asynchronous population and allowed synthesis of new protein during 7 hours. The newly synthesized pool of CENP-N-SNAP was then fluorescently labeled with TMR-*Star* and cells were fixed and processed for immunofluorescence. If CENP-N was assembled in a tight specific cell cycle window like CENP-A, one would expect that only a proportion of the cells was labeled with centromeric TMR-*Star*. However, the majority of the cells, except metaphase cells, were TMR-*Star* positive (Figure 3.2), indicating that CENP-N assembly occurs throughout most of the cell cycle. In addition, we observed that CENP-N assembly occurs mainly during late G1/S and G2 phases and decreases in prometaphase [based on cyclin B levels and microtubule staining patterns as a measure of cell cycle position (Figure 3.2)]. This result is consistent with previous reports of a severe decrease in CENP-N levels during metaphase and an increase upon mitotic exit

(McClelland et al., 2007). In summary, we have shown that CENP-N targeting to centromeres, unlike CENP-A assembly, is not restricted to G1 phase of the cell cycle. Instead, this CCAN component that binds directly to CENP-A nucleosomes appears to be assembled at centromeres during different phases of the cell cycle.

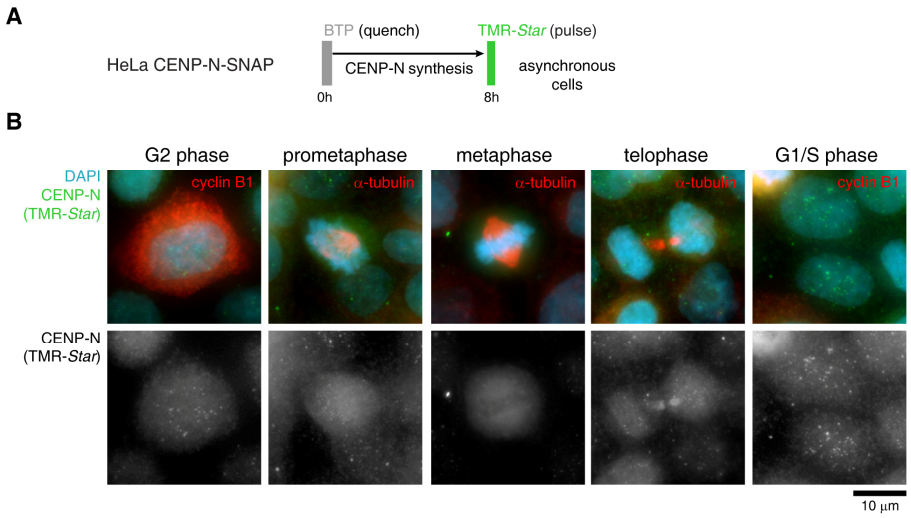

Figure 3.2. CENP-N targeting to centromeres is not restricted to G1 phase. (A) An asynchronous population of HeLa cells expressing CENP-N-SNAP were quench-chase-pulse labeled (as described in Chapter 2) to assess the timing of CENP-N targeting to centromeres. (B) Representative images of HeLa CENP-N-SNAP cells in the different stages of the cell cycle. Cells were counterstained for CENP-T to mark the centromeres, cyclin B1 to indicate G2 status (when positive) and G1/S status (when negative), α-tubulin to distinguish the different mitotic stages, and with DAPI to visualise DNA.

Although this protein showed a dynamic localization at centromeres, we cannot exclude the possibility that a subpopulation of CENP-N remains stably bound to centromeres during a specific cell cycle window. Indeed, a recent study demonstrated that CENP-N undergoes rapid exchange during G1 until the middle of S phase when it becomes stably associated with centromeres (Hellwig et al., 2011). CENP-C, the other CCAN protein able to bind directly to CENP-A nucleosomes (Carroll et al., 2010), is recruited to centromeres during anaphase

along with CENP-A^{CID} in *D. melanogaster* embryos (Schuh et al., 2007). In human cells, CENP-C exhibits dynamic exchange throughout the majority of the cell cycle and becomes stably bound to centromeres only during mitosis and S phase (Hemmerich et al., 2008). These results show that, while assembly of CENP-A is tightly cell cycle restricted (Jansen et al., 2007), the proteins that directly bind to CENP-A nucleosomes are more dynamic. Therefore, the cell cycle coupling observed for CENP-A assembly is probably unique among centromere proteins and is the subject of further investigation in this chapter.

Cdk inhibition triggers CENP-A assembly prior to mitosis

To indentify the molecular mechanism controlling the unusual timing of CENP-A assembly, we used the previously established HeLa cell line that stably expresses CENP-A-SNAP (Jansen et al., 2007), and specifically labelled the nascent pool of this protein. Assembly of CENP-A quickly follows mitotic exit but, as described above, multiple processes occur during mitosis that could trigger this event. From among several mitotic signals, it was previously shown that neither microtubule attachment nor microtubule-generated tension across centromeric chromatin is required for normal CENP-A assembly. This was achieved by depolymerizing microtubules using the spindle poison Nocodazole. As this results in a mitotic checkpoint-dependent cell cycle arrest, these cells were forced to exit mitosis by simultaneously depletion of mitotic checkpoint proteins (Jansen et al., 2007; Schuh et al., 2007). However, because these results were obtained in the absence of mitotic checkpoint proteins, they did not test for the involvement of these proteins in preventing CENP-A assembly. The requirement for kinetochore-microtubule attachments and checkpoint inactivation for mitotic exit can be circumvented by direct inhibition of Cdk1 activity, using small molecule inhibitors such as Roscovitine. Therefore, we decided to determine whether the presence mitotic checkpoint proteins would prevent CENP-A assembly under conditions where the checkpoint is activated. To test

this we synchronized cells in mitosis with Colcemid to depolymerize microtubules, labeled a newly synthesized pool of CENP-A-SNAP and forced cells to exit mitosis using Roscovitine. Roscovitine treated cells successfully exited mitosis without chromosome segregation and cytokinesis due to the absence of microtubules, indicating effective inhibition of Cdk activity. The resulting tetraploid cells loaded CENP-A at centromeres normally (Figure 3.3). These results indicate that kinetochore-microtubule attachments are not required for subsequent CENP-A assembly, either in the presence or absence of checkpoint proteins. While mitotic checkpoint proteins are presumably not removed under these conditions we cannot exclude the possibility that checkpoint activity is impaired. Roscovitine treatment induces dissociation of Mad2 from Cdc20 (D'Angiolella et al., 2003; Visconti et al., 2010), which normally occurs when the checkpoint is satisfied. However, the mitotic checkpoint kinase BubRI remains active under these conditions in *X. leavis* egg extracts (Mao et al., 2005). This suggests that the mitotic checkpoint may be partially active after Cdk1 inhibition by Roscovitine, but that this does not interfere with CENP-A assembly.

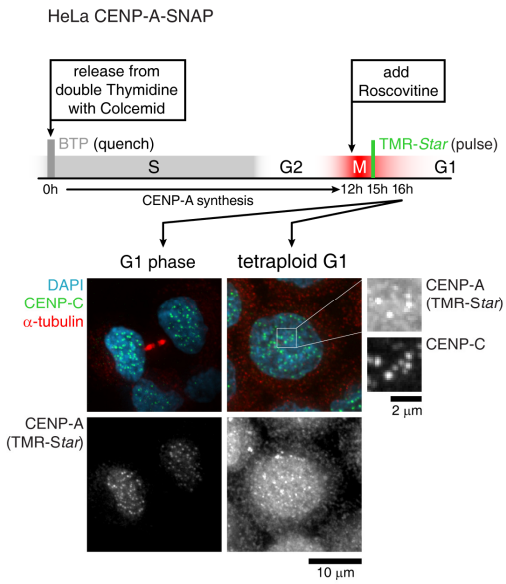

Figure 3.3. Cdk1 inhibition in the presence of checkpoint proteins does not interfere with CENP-A assembly. HeLa CENP-A-SNAP cells were synchronized in mitosis with a double Thymidine block followed by treatment with Colcemid. Mitotic cells were either treated with Roscovitine for 4 hours (tetraploid G1) or released from the Colcemid block and allowed to progress through mitosis normally (G1 control). Prior to fixation, a nascent pool of CENP-A-SNAP was pulse labelled with TMR-*Star*. Cells were counterstained for α-tubulin, CENP-C and with DAPI to indicate G1 status, centromeres and DNA, respectively.

Another defining feature of mitotic exit, downstream of mitotic checkpoint satisfaction, is APC/C activation. APC/C mediates degradation of several mitotic proteins (see Chapter 1, Section 4.2.2), including cyclin B, which leads to loss of Cdk activity. We hypothesized that CENP-A assembly might be controlled directly by Cdk activity without the need for checkpoint satisfaction or any other aspect of mitosis. To test this hypothesis, we synchronized cells in G2 phase and fluorescently pulse labeled a nascent pool of CENP-A-SNAP, synthesized during the preceding S phase. We then treated the cells for 1 hour with either Roscovitine or Purvalanol A, two pan-Cdk inhibitors, and processed cells for immunofluorescence (Figure 3.4 A and F). Strikingly, this brief 1 hour treatment was sufficient to induce CENP-A assembly into centromeres in nearly half of the cyclin B positive population, which are mainly G2 cells. In untreated cells, CENP-A assembly was only observed during late telophase/early G1 phase and the nascent pool of CENP-A-SNAP remained diffusely nuclear in G2 phase (with a slight accumulation in the nucleolus) (Figure 3.3 A-C, F and G).

Interestingly, we observed that a Roscovitine treatment as short as 30 minutes was sufficient to trigger CENP-A assembly prior to mitosis, suggesting that the CENP-A assembly machinery is present throughout cell cycle and can be rapidly activated (Figure 3.4 D). This induction of CENP-A assembly in G2 phase by Roscovitine is likely incomplete as physiological CENP-A assembly continues for the duration of G1 phase, which takes approximately 10 hours (Lagana et al., 2010). Consistent with this idea, the levels of CENP-A assembled in G2 centromeres appeared slightly lower when compared with the levels assembled in G1 centromeres (Figure 3.4 E). In addition, the number of G2 cells loading CENP-A increased with the extension of Roscovitine treatment (Figure 3.4 D), which also led to more apoptosis [6% or 1% of apoptotic cells were observed when cells were treated with Roscovitine for 4 or 1 hour, respectively (Wojciechowski et al., 2003; Wesierska-Gadek et al., 2008)]. Thus, in subsequent experiments, cells were treated with this Cdk inhibitor for only 1 hour.

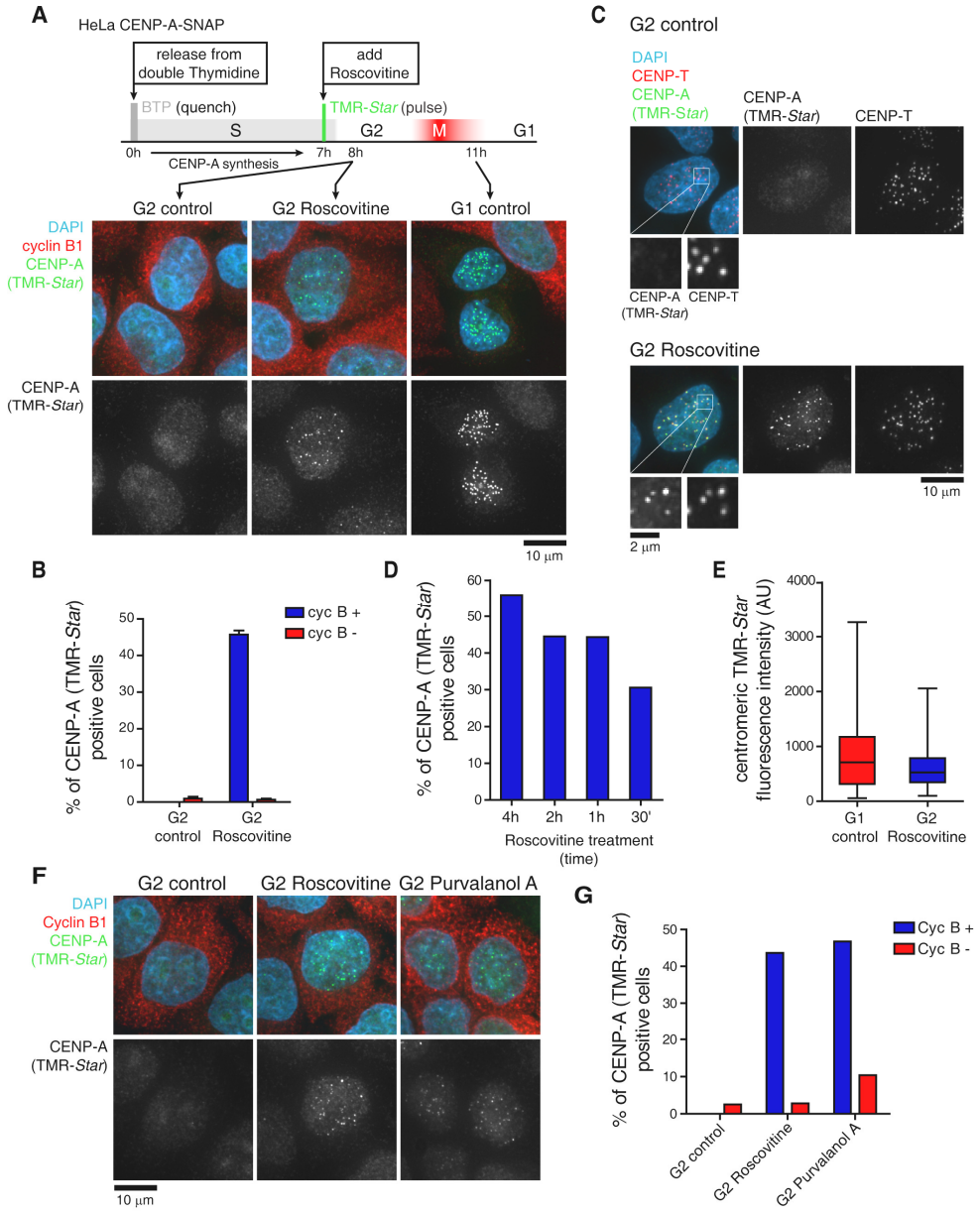

Figure 3.4. Roscovitine or Purvalanol A treatment induces CENP-A assembly at centromeres in G2 phase. (A) HeLa CENP-A-SNAP cells were synchronized at the G1/S boundary by double Thymidine arrest. S phase synthesized CENP-A-SNAP was subsequently pulse labeled in G2 phase, 7 hours after release from Thymidine. G2 cells were either mock treated (G2 control), treated with Roscovitine for 1 hour (G2 Roscovitine) or allowed to cycle through mitosis (G1 control) prior to fixation. Continued on the next page.

Figure 3.4. (continued) Cells were counterstained for cyclin B and with DAPI to indicate G2 status and DNA, respectively. (B) Quantification of A. Mean and standard error of the mean (SEM) of 3 replicates are shown. ~200 cells were analyzed in each replicate. (C) Nascent CENP-A-SNAP colocalizes with centromeres (CENP-T) after Roscovitine induced assembly in G2 phase HeLa CENP-A-SNAP cells. (D) Experiment as in A, except that cells were treated with Roscovitine during different periods of time: 30 min, 1, 2 or 4 hours. (E) Box and whisker plots of relative CENP-A-SNAP TMR-*Star* fluorescent signal per centromere in G2 Roscovitine treated cells and in G1 control cells. CENP-T was used as a reference for centromere position. More than 500 centromeres were quantified in each condition. (F) Experiment as in A, but G2 cells were treated with either Roscovitine or Purvalanol A. (G) Quantification of F. ~ 100 cells were analyzed. In B and G the percentage of total cells positive for centromeric CENP-A-SNAP (TMR-*Star*) signal was scored and represented according to cyclin B status.

We confirmed that Cdk inhibition is sufficient to triggers CENP-A assembly during G2 phase in nontransformed telomerase-expressing retinal pigment epithelial cells (hTERT-RPE). For this, we treated randomly cycling hTERT-RPE cells expressing CENP-A-SNAP with Roscovitine for 1 hour and observed that 94% of the G2 cells, marked by separated centrosomes, incorporated nascent CENP-A-SNAP into their centromeres (Figure 3.5).

To test whether Roscovitine induced assembly in G2 phase resulted in stable incorporation of CENP-A into centromeric chromatin, we examined if the nascent pool of CENP-A-SNAP is retained through mitosis. We treated cells as indicated in Figure 3.4 A, except that Roscovitine was washed out after 1 hour treatment and cells were subsequently released in complete medium for 1 hour, before fixation, to allow them to enter mitosis. We found that CENP-A is stably retained at centromeres on condensed mitotic chromosomes following Roscovitine washout (Figure 3.6), strongly suggesting that Cdk inhibition is sufficient to activate all the three steps of CENP-A assembly: licensing, loading, and stabilization (see Chapter 2).

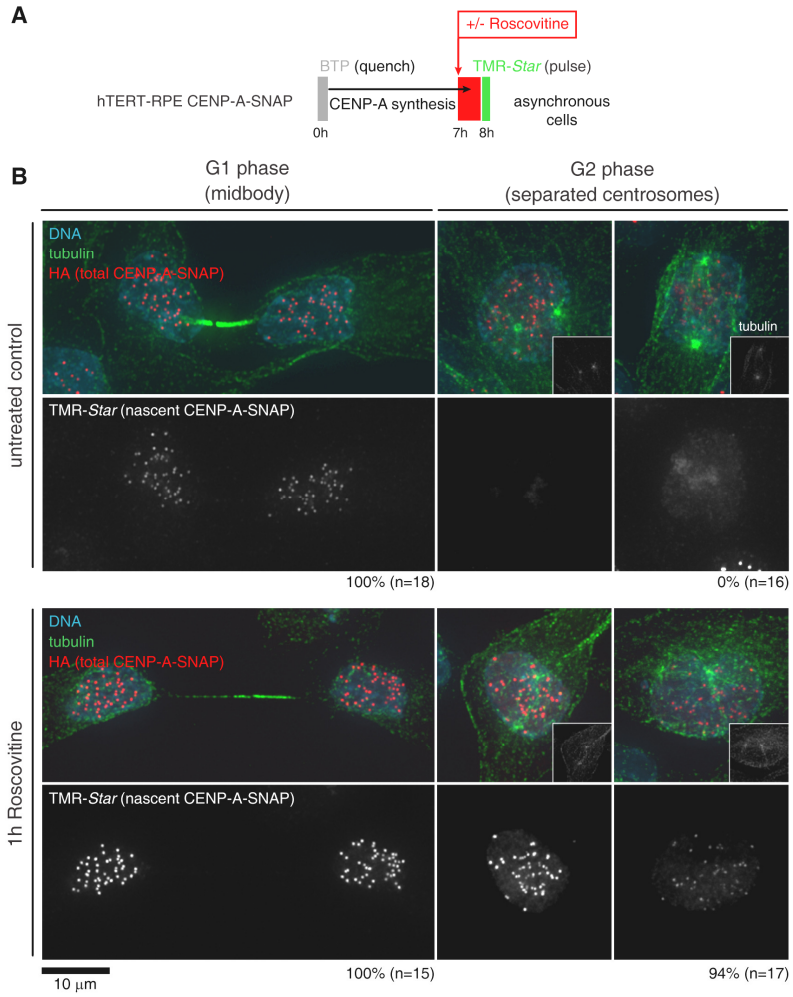

Figure 3.5. G1 phase assembly of CENP-A-SNAP and G2 induction by Roscovitine in hTERT-RPE cells. (A) Randomly cycling hTERT-RPE cells stably expressing CENP-A-SNAP were subjected to quench-chase-pulse labeling. Cells were either mock treated (G2 control) or treated with Roscovitine for 1 hour (G2 Roscovitine) prior to fixation (B) Newly synthesized fluorescently labeled CENP-A-SNAP (TMR-*Star*) localized to centromeres in early G1 phase cells (as identified by midbody staining) but not to G2 phase cells (marked by separated centrosomes). A 1 hour treatment with Roscovitine prior to fixation was sufficient to induce CENP-A-SNAP assembly at centromeres in the majority of G2 cells (94%). Two examples of G2 cells are shown for each condition. Cells were counterstained for HA to visualize the total (preincorporated and nascent) pool of CENP-A-SNAP, for tubulin to indicate cell cycle status and with DAPI for DNA.

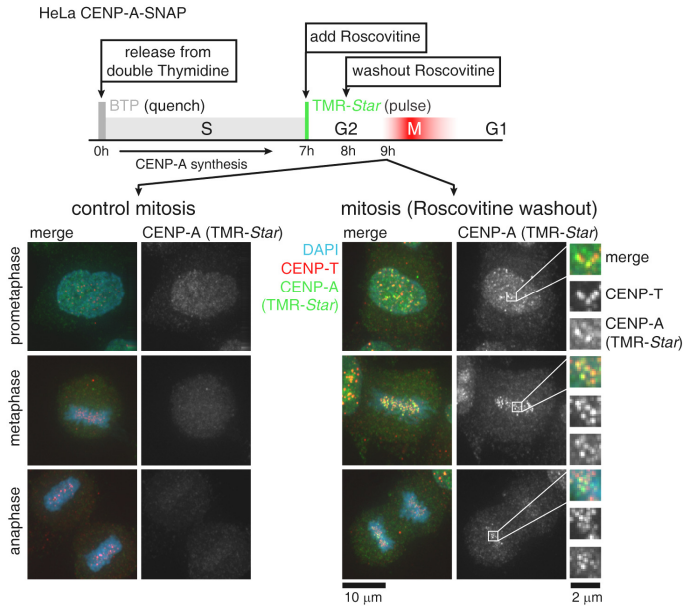

Figure 3.6. CENP-A assembled in G2 phase is stable during mitosis. Experiment as in Figure 3.4 A but cells were released from Roscovitine after a 1 hour induction of CENP-A assembly in G2 phase. Cells were analyzed in mitosis (1 hour after washout) and scored for retention of nascent CENP-A-SNAP on mitotic chromosomes. Cells were counterstained for CENP-T and with DAPI to visualize centromeres and DNA, respectively.

DNA replication and CENP-A assembly are mutually exclusive in human cells

Whereas the assembly of histone H3.1 (the canonical histone H3), is coupled with DNA replication, the assembly of the variants H3.3 and CENP-A occurs in a DNA synthesis-independent manner (Shelby et al., 2000; Tagami et al., 2004). Although histone H3.3 assembly is largely uncoupled from DNA replication, this histone variant can be incorporated into chromatin both during and outside of S phase (Ahmad and Henikoff, 2002; Ray-Gallet et al., 2011). To determine whether CENP-A deposition can occur during S phase, simultaneously with H3.1 assembly, we synchronized cells in late S phase or G2 phase by

double Thymidine block followed by a 5 or 7 hours release, respectively. Following synchronization, Cdk activity was inhibited using Roscovitine. While a significant fraction of cells enriched in G2 phase assembled nascent CENP-A-SNAP at centromeres, only a small percentage of cells enriched in late S phase did so (Figure 3.7).

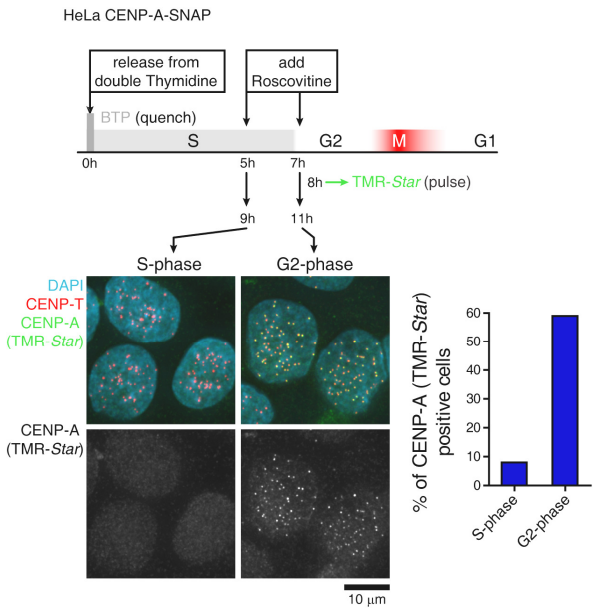

Figure 3.7. Cdk inhibition triggers CENP-A loading in cells enriched in G2 phase but not in those enriched in S phase. HeLa CENP-A-SNAP cells were synchronized at the G1/S boundary by double Thymidine arrest. Cells were enriched in S and G2 phase by release from Thymidine for 5 and 7 hours, respectively. The distinct populations of cells were treated with Roscovitine for 4 hours and a nascent pool of CENP-A-SNAP was pulse labeled before fixation. Cells were counterstained for CENP-T and with DAPI to indicate centromeres and DNA, respectively. ~ 100 cells were analyzed.

This result suggests that S phase is refractory to CENP-A assembly even upon Roscovitine induction. Since synchrony is inherently imperfect, the fraction of cells that assembled CENP-A-SNAP in the S phase enriched condition may already have progressed into G2 phase. To confirm that CENP-A assembly cannot occur in S phase we repeated the SNAP-based quench-chase-pulse experiment described above with BrdU labeling to visualize S phase cells. Incorporation of nascent CENP-A-SNAP was never observed simultaneously with positive BrdU staining, whereas it was readily observed in the majority of the BrdU negative cells (Figure 3.8). This result strongly suggests that cells actively

replicating DNA are unable to assemble CENP-A, despite Cdk inhibition. A 7 hour release from a double Thymidine block resulted in approximately 40% of BrdU positive cells, which are unable to load CENP-A. This explains why only nearly 50% of cyclin B positive cells assembled CENP-A in the experiment represented in Figure 3.4 A-D.

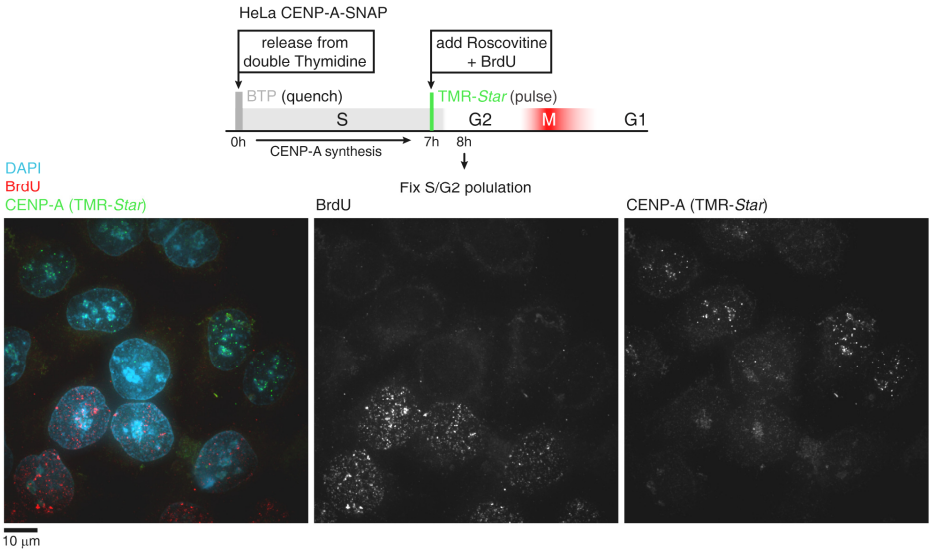

Figure 3.8. S phase cells are refractory to CENP-A assembly. (A) HeLa CENP-A-SNAP cells were synchronized as described in Figure 3.4 A resulting in population that contains both S and G2 phase cells. S phase synthesized CENP-A-SNAP was pulse labeled 7 hours after release from Thymidine. Cells were then treated for 1 hour with Roscovitine and BrdU, which labels cells actively undergoing DNA replication (S phase). Cells were counterstained using an anti-BrdU antibody and DAPI, to indicate DNA replication and DNA, respectively.

APC/C mediated proteolysis and protein synthesis are not required for CENP-A assembly in G2-phase.

CENP-A assembly into centromeric chromatin requires loss of Cdk activity upon mitotic exit, which occurs through APC/C^{Cdc20}-mediated degradation of the Cdk1 activator cyclin B. When the mitotic checkpoint is active, the mitotic checkpoint complex (MCC) interacts with the APC/C activator Cdc20, preventing APC/C activation (see Chapter 1, Section 2.4.1; Herzog et al., 2009). This MCC/Cdc20 interaction requires continued Cdk1 activity (D'Angiolella et al., 2003). Treatment of mitotic cells with Roscovitine therefore results in premature activation of APC/C^{Cdc20} (Listovsky et al., 2000). This indicates that Roscovitine-induced CENP-A assembly may be a consequence of APC/C^{Cdc20} mediated degradation of an inhibitor of CENP-A assembly.

To test whether Roscovitine also induces APC/C^{Cdc20} activation in G2 phase, we treated G2 synchronized cells with this inhibitor and examined the levels of cyclin B by immunobloting and immunoflurescence. Cyclin B levels remained high by either measure (Figures 3.4 A and F and 3.9 A and C), indicating that Roscovitine treated cells do not enter a precocious G1-like state by premature activation of APC/C^{Cdc20} mediated proteolysis. Indeed, blocking overall proteolysis with the proteosome inhibitor MG132 did not abolish G2-induced CENP-A assembly (Figures 3.9 A, B and E). Similar results were obtained after treatment with Cycloheximide, demonstrating that *de novo* synthesis of proteins is also not required for unscheduled CENP-A assembly (Figure 3.9 A, B and D). These results argue against a role for APC/C-mediated degradation of a putative inhibitor of CENP-A assembly other than the Cdk activator cyclin B. Moreover, these observations suggest that the CENP-A assembly machinery is already present and poised for activation in G2 phase without the need for new synthesis of any activator of the CENP-A assembly pathway.

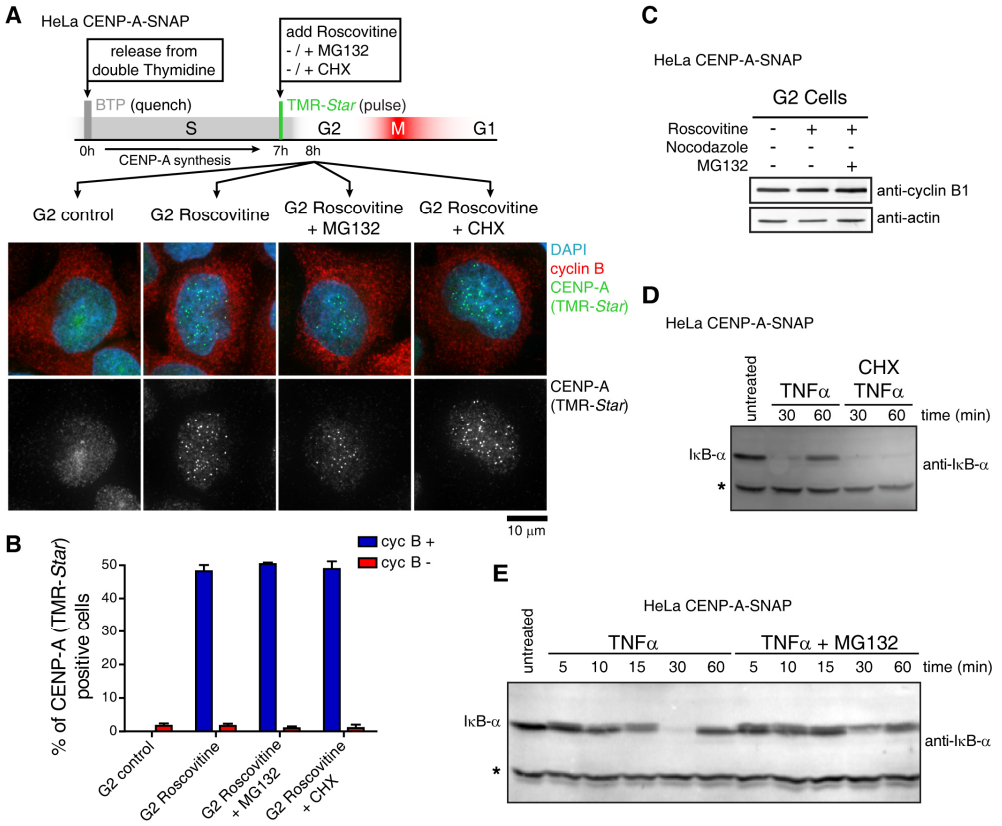

Figure 3.9. Neither APC/C mediated proteolysis nor protein synthesis is required for CENP-A assembly in G2 phase. (A) HeLa CENP-A-SNAP cells were synchronized in G2 phase and were subjected to quench-chase-pulse labeling as in Figure 3.4 A. G2 cells were treated for 1 hour with either Roscovitine alone or with Roscovitine in combination with MG132 or Cycloheximide (CHX) to block proteolysis or protein synthesis, respectively. Cells were counterstained for cyclin B and with DAPI to indicate G2 status and DNA, respectively. (B) Quantification of A. Mean and standard error of the mean (SEM) of 3 replicates are shown. Percentage of total cells positive for centromeric CENP-A-SNAP (TMR-*Star*) signal was scored and represented according to cyclin B status. (C) G2 phase cyclin B levels remain unchanged after MG132 and/or Roscovitine treatment at conditions used in A. HeLa CENP-A-SNAP cells were synchronized in G2 as in A followed by a 1 hour treatment with indicated drugs. Cells were processed for immunoblot and probed for cyclin B1 levels or actin (as a loading control). Continued on the next page.

Figure 3.9. (continued) (D and E) TNFα induces rapid proteolytic degradation of IκB-α which is followed by rapid de novo synthesis (Seldon et al., 2007). MG132 prevents TNFα induced IκB-α degradation while Cycloheximide prevents resynthesis. HeLa CENP-A-SNAP cells were either treated with TNFα alone or along with Cycloheximide (CHX) (D) or MG132 (E) for up to 60 minutes. IκB-α levels were determined by immunoblot at indicated time points. Asterisk indicates a cross reacting band used as a loading control.

The efficiency of MG132 and Cycloheximide was determined using an assay that allows for the measurement of changes in the rate of protein degradation or synthesis within one hour. We used TNFα induced degradation of IκB-α that occurs within minutes following TNFα addition (Figure 3.9 E and Seldon et al., 2007). IκB-α resynthesis is in turn very fast and its levels come back up within an hour (Figure 3.9 D and Seldon et al., 2007). Using this system we validated MG132 and Cycloheximide activity. We observed that MG132 prevents degradation of IκB-α upon TNFα addition (Figure 3.9 E), and Cycloheximide prevents IκB-α resynthesis (Figure 3.9 D) within the short timescales used in our experiments.

Cdk1 inhibition is not sufficient to induce CENP-A assembly in G2 phase.

The predominant Cdks that are active during G2 phase and mitosis are Cdk2 and Cdk1, respectively. These kinases are naturally deactivated upon mitotic entry and mitotic exit by APC/C-mediated degradation of cyclin A and cyclin B, respectively (den Elzen and Pines, 2001; Clute and Pines, 1999). Roscovitine and Purvalanol A are potent inhibitors of both Cdk1 and Cdk2 (De Azevedo et al., 1997; Meijer et al., 1997), and are sufficient to induce CENP-A assembly prior to mitotic exit. However, due to the broad substrate specificity of these inhibitors (Wesierska-Gadek and Krystof, 2009), it is difficult to determine which Cdk (if any) is responsible for controlling CENP-A loading. To test if Cdk1 inhibition is sufficient to induce CENP-A assembly prior to mitosis we preformed a

similar experiment to the one represented in Figure 3.4 A, except that we treated cells with RO3306, a specific inhibitor of Cdk1 (Vassilev et al., 2006). Due to premature loss of Cdk1 activity, mitotic cells treated with this specific inhibitor exited mitosis without chromosome segregation and cytokinesis and, as a result, they assembled CENP-A at centromeres (tetraploid G1 in Figure 3.10). In contrast, CENP-A assembly was not observed in G2 phase RO3306 treated cells, indicating that Cdk1 inhibition is not sufficient to trigger unscheduled centromere propagation (G2 RO3306 in Figure 3.10).

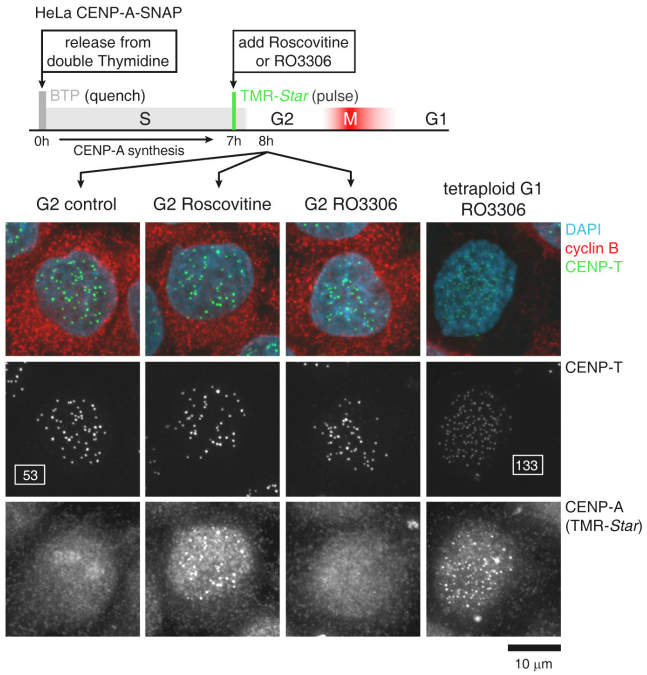

Figure 3.10. Cdk1 inhibition is not sufficient to trigger CENP-A assembly in G2 phase. HeLa CENP-A-SNAP cells were synchronized in G2 phase and were subjected to quench-chase-pulse labeling as in Figure 3.4 A. G2 cells were either treated with Roscovitine or with the specific Cdk1 inhibitor, RO3306. Cells were counterstained for cyclin B, CENP-T and with DAPI to indicate G2 status, centromeres and DNA, respectively. The boxed numbers in the CENP-T images indicate the centromere number per cell (a tetraploid cell presents approximately twice as many centromeres than a G2 control cell, indicating that chromosome segregation and cytokinesis did not occur in this cell).

Cdk1 and Cdk2 activities are sufficient to maintain cell cycle control of CENP-A assembly

Our small molecule inhibitor experiments demonstrated that inhibition of Cdk1 activity alone was not enough to drive CENP-A assembly. Cdk2 is also unlikely to individually regulate CENP-A assembly because it is inactivated in early mitosis, through APC/C^{Cdc20}-mediated degradation of cyclin A (Geley et al., 2001; den Elzen and Pines, 2001), while CENP-A assembly occurs only later upon mitotic exit, concomitant with proteolysis of cyclin B [Figure 3.4 A and (Jansen et al., 2007; Hemmerich et al., 2008)]. Thus, CENP-A assembly is likely controlled by both Cdk1 and Cdk2, which are known to present some redundant functions and are both active prior to mitotic exit (Hochegger et al., 2008). To test this hypothesis directly we abandoned the use of small molecule inhibitors, such as Roscovitine, because of their broad substrate specificity and toxicity (Savio et al., 2006; Wesierska-Gadek et al., 2008; Wesierska-Gadek and Krystof, 2009). Instead, we decided to directly test the roles of Cdk1 and Cdk2 by genetic means.

Cdk2 is a nonessential gene in mice and DT40 cells, allowing us to test its specific role in CENP-A assembly by gene deletion (Berthet et al., 2003; Ortega et al., 2003; Hochegger et al., 2007). Conversely, Cdk1 is an essential protein, preventing the use of null alleles. Instead, we used chemical genetic tools, which have recently been developed to allow highly selective inhibition of kinase activity. This is achieved by targeted mutations to a conserved bulky residue in the active site of a kinase, such as Cdk1, which renders it sensitive to a specific bulky ATP analog (Bishop et al., 2001; Shokat and Velleca, 2002). Importantly, these ATP analogs are not recognized by any endogenous kinase. Therefore, this approach creates a conditional kinase whose inhibition is specific, fast and reversible.

To test which Cdk (if any) is responsible for controlling the timing of CENP-A assembly we turned to chicken DT40 cells that contain defined mutations in

Cdk2 and/or Cdk1 [developed by Helfrid Hochegger, University of Sussex, Brighton, UK; (Hochegger et al., 2007)]. We used cells that express an analog-sensitive conditional mutant of Cdk1 in a homozygous *cdk1* null background (*cdk1as*) or in a homozygous *cdk1* and *cdk2* null background (*cdk1as/cdk2*$^{-/-}$) (Hochegger et al., 2007). Cdk1as can be selectively and reversibly inhibited by addition of the ATP analog 1NM-PP1. 1NM-PP1 does not affect cell cycle progression of wild-type DT40 cells, highlighting the specificity of this molecule to the cdk1as mutation (Figure 3.11 A and Hochegger et al., 2007). Low concentrations (1 µM) of 1NM-PP1 result in partial inhibition of the analog-sensitive version of Cdk1 (CDK1as), while high concentrations (10 µM) lead to its full inhibition (Hochegger et al., 2007). Treatment of *cdk1as* single mutants with low (1 µM) or high (10 µM) concentrations of 1NM-PP1 results in G2 phase arrest, as cells require high Cdk1 activity to enter mitosis (Figure 3.11 A). In the absence of *CDK2*, cyclins A and E can activate Cdk1 and enable it to compensate for the loss of Cdk2 (Berthet et al., 2003; Ortega et al., 2003; Aleem et al., 2005; Hochegger et al., 2007; Santamaría et al., 2007; Krasinska et al., 2008). As a result, *cdk1as/cdk2*$^{-/-}$ double mutants arrest in S phase when treated with 1NM-PP1 concentrations sufficient to fully inhibit Cdk1as. In contrast, partial Cdk1as activity (1 µM 1NM-PP1) is sufficient to drive S phase progression but not high enough to allow entry into mitosis (Figure 3.11 A and Hochegger et al., 2007).

Sequence alignments show that chicken CENP-A shares 56% amino acid homology with its human homologue (Figure 3.11 B). The CENP-A targeting domain (CATD) is highly similar between these two homologues (80% amino acid homology), which indicates that the CENP-A assembly process may occur through a similar mechanism in these two organisms. To assay CENP-A assembly in DT40 cells, we created both *cdk1as* single and *cdk1as/cdk2*$^{-/-}$ double mutant clones that stably express SNAP-tagged chicken CENP-A. We selected clones that expressed this fusion protein at subendogenous levels and displayed a centromeric fluorescent signal following pulse labeling with TMR-*Star* (Figure

3.1 in Materials and Methods and 3.11 C and C'). To test if the timing of CENP-A assembly is conserved between human and chicken DT40 cells we used the quench-chase-pulse labeling strategy analogous to the one described for HeLa cells in Chapter 2. We labeled the nascent pool of chicken SNAP-CENP-A with TMR-*Star* in either asynchronous or mitotic arrested populations in both *cdk1as* single and *cdk1as/cdk2*$^{-/-}$ double mutants (Figure 3.11 C). In a randomly cycling population, approximately 40 % of the cells loaded SNAP-CENP-A at centromeres, indicating that CENP-A assembly does not occur throughout the cell cycle, but is restricted to a specific cell cycle window (Figure 3.11 C-D). Importantly, mitotically arrested DT40 cells did not assemble SNAP-CENP-A at centromeres (Figure 3.11 C-D). This demonstrates that, like in human cells [Figure 3.4 A and (Jansen et al., 2007; Hemmerich et al., 2008)], *Drosophila melanogaster* embryos (Schuh et al., 2007) and *Xenopus leavis* extracts (Bernad et al., 2011; Moree et al., 2011), chicken DT40 cells assemble CENP-A at centromeres only upon mitotic exit (Figure 3.11 C-D). The timing of assembly was identical in *cdk1as* single and in *cdk1as/cdk2*$^{-/-}$ double mutant cells (Figure 3.11 C-D), demonstrating that inhibition of Cdk2 alone is not sufficient to induce unscheduled CENP-A assembly.

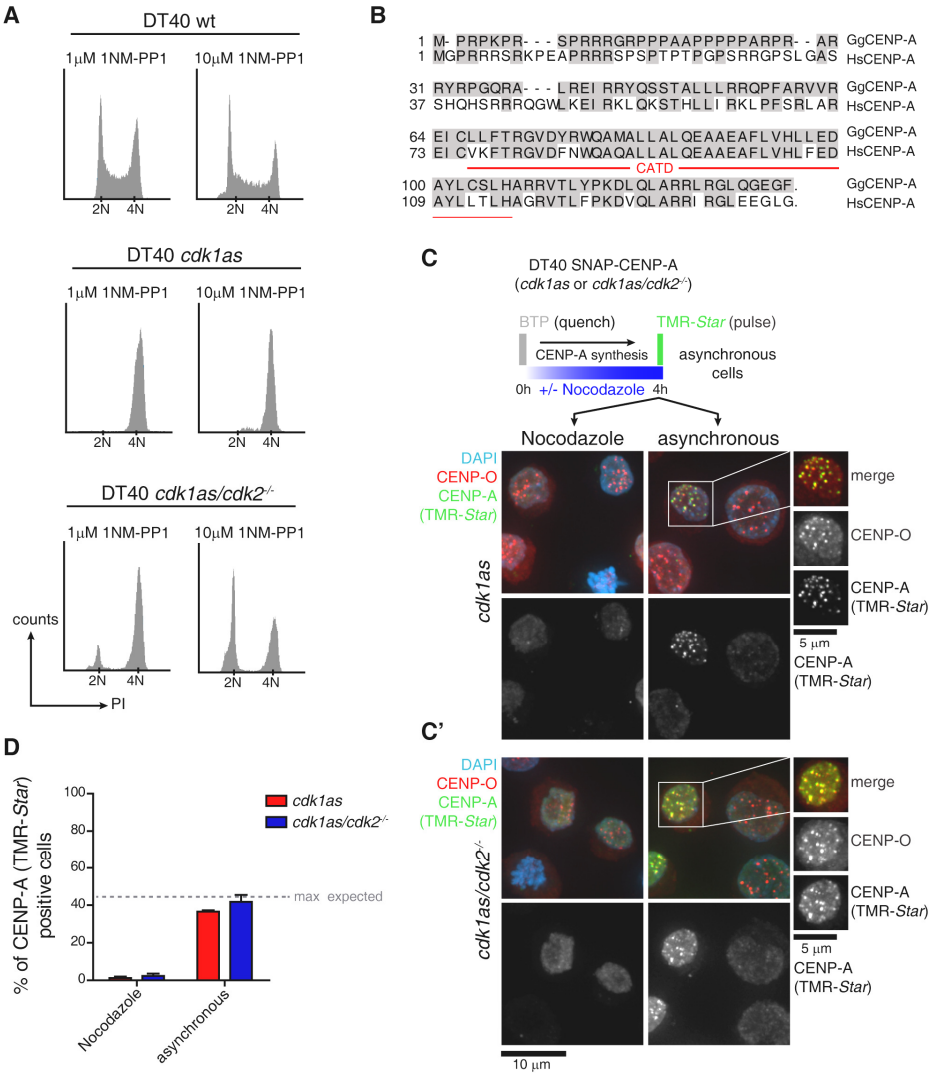

Figure 3.11. The timing of CENP-A assembly is conserved in DT40 cells. (A) DT40 *cdk1as* single or *cdk1as/cdk2*$^{-/-}$ *double* mutant cells were treated with either low (1 μM) or high (10 μM) 1NM-PP1 for 12 hours. Cells were fixed and counterstained with propidium iodide (PI) to mark the DNA, and analyzed by flow cytometry. (B) Sequence alignment of CENP-A homologues from *Gallus gallus* (chichen) and *Homo sapiens* (human). Shading indicates identical residues. CENP-A targeting domain (CATD) is underlined in Red. Continued in the next page.

Figure 3.11. (continued) (C) DT40 *cdk1as* single mutant cells (i.e. active Cdk1 and Cdk2) stably expressing SNAP-CENP-A were assayed for assembly of a nascent pulse labeled pool of SNAP-CENP-A in either asynchronous cultures or cells prevented from entering G1 phase through treatment with Nocodazole, a microtubule depolymerizing drug. Insets show nascent SNAP-CENP-A (TMR-*Star*) colocalization with centromeres (CENP-O). (C´) as C but for *cdk1as/cdk2*$^{-/-}$ double mutant cells (i.e. Cdk2 null but active Cdk1). (D) Quantification of C and C´. Dotted line represents the maximally expected percentage of cells assembling CENP-A [3,5 hours synthesis / 8 hours cell cycle (Zhao et al., 2007) x 100 = 44%]. Mean and SEM of 3 replicates of each condition are shown. ~ 150 cells were analyzed for each replicate

Once we established that the timing of CENP-A incorporation is conserved in human and DT40 cells, we determined whether our results of Roscovitine-induced assembly during G2 phase in human cells could be recapitulated in DT40 cells. To test this, we synchronized *cdk1as* single mutant cells in G2 phase with low levels of 1NM-PP1 (1 µM), labeled a nascent SNAP-CENP-A pool, and treated cells with Roscovitine for 4 hours. Consistent with our observations in HeLa cells, Roscovitine triggered centromeric chromatin assembly prior to mitosis in DT40 cells (Figure 3.12 A and B). We observed that close to 100% of Roscovitine treated cells assembled CENP-A at G2 centromeres (Figure 3.12 B), showing that in these cells Roscovitine treatment is more effective than in HeLa cells. This could be the result of the better G2 synchronization achieved with the 1NM-PP1 treatment.

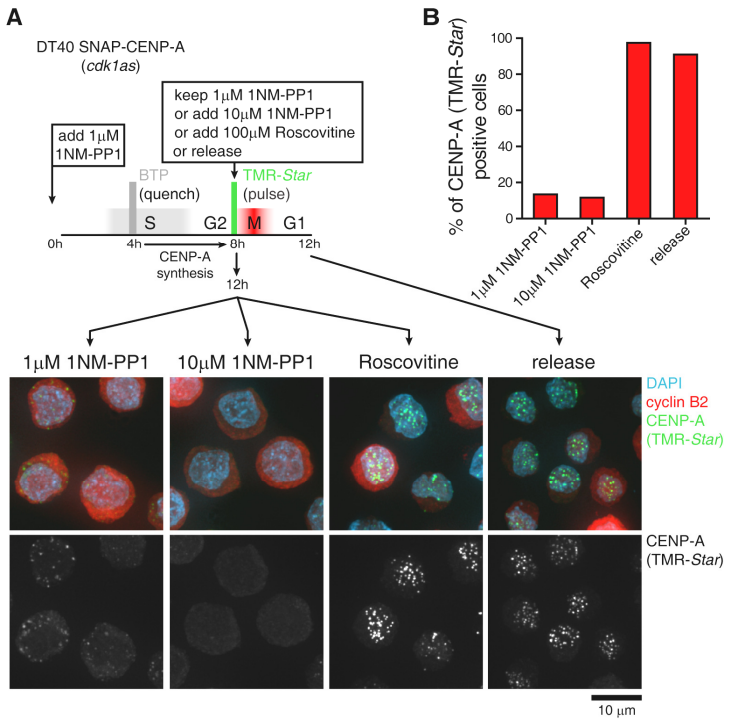

Figure 3.12. Roscovitine induces CENP-A assembly at centromeres during G2 phase in DT40 cells. (A) DT40 *cdk1as* cells were synchronized in G2 phase with a low concentration (1 μM) of 1NM-PP1, followed by labeling of a nascent SNAP-CENP-A pool with TMR-*Star*. Cells were then either kept arrested in G2 with low levels of 1NM-PP1 (1μM), high levels of 1NM-PP1 (10μM), or with Roscovitine or released into G1 followed by fixation and processing for imaging. Cells were counterstained for cyclin B2 and with DAPI to indicate G2 status and DNA, respectively. (B) Quantification of A. ~ 100 cells were analyzed.

We demonstrated that individual inhibition of either Cdk1 or Cdk2 is not sufficient to trigger unscheduled CENP-A assembly by using a specific small molecule inhibitor of Cdk1 in HeLa cells (Figure 3.10) and through *CDK2* gene deletion in DT40 cells (Figure 3.11 C'), respectively. One possible explanation for these results is that both these kinases are involved in controlling the timing of centromere propagation. Indeed, they are both active during G2 phase, they have known overlapping functions (Satyanarayana and Kaldis, 2009; Pagliuca et al., 2011), and they are both inhibited by Roscovitine (Wesierska-Gadek and Krystof, 2009). An alternative explanation is that CENP-A loading is controlled by other

Roscovitine targets, such as Cdk5, Cdk7 and/or Cdk9 (Bach et al., 2005). To distinguish between these two hypotheses, we specifically inhibited Cdk2 and/or Cdk1, in *cdk1as* single and *cdk1as/cdk2*$^{-/-}$ double mutant cells using the ATP analog 1NM-PP1. We synchronized *cdk1as* single or *cdk1as/cdk2*$^{-/-}$ double mutant cells in G2 phase with low levels of 1NM-PP1 (1 μM) and labeled a newly synthesized SNAP-CENP-A pool. Cell were either maintained in G2 in low inhibitor concentrations, or shifted to high (10 μM) doses of ATP analog to completely abolish Cdk1 activity, or released into G1 phase by removing the inhibitor (Figure 3.13 A). While centromeric CENP-A assembly was detected in G1 phase in cells of either genotype, *cdk1as* single mutants showed little centromere assembly in G2 (Figures 3.13 A and B). Strikingly, G2 arrested *cdk1as/cdk2*$^{-/-}$ double mutant cells readily incorporated CENP-A at centromeres in practically all cells in both low and high doses of 1NM-PP1 (Figures 3.13 A' and B). The levels of centromeric SNAP-CENP-A in G2 *cdk1as/cdk2*$^{-/-}$ cells in which Cdk1 was partially inhibited were comparable to the SNAP-CENP-A centromeric levels in G1 cells (Figure 3.13 C). This suggests that, in contrast to the partial assembly observed in Roscovitine-treated HeLa cells, inhibition of Cdk1 and Cdk2 can induce complete CENP-A incorporation prior to mitosis. This could be a result of the shorter cell cycle in DT40 cells. The cell cycle duration of DT40 cells is approximately 8 hours with a G1 phase of approximately 1 to 2 hours (Hochegger et al., 2007; Zhao et al., 2007). Therefore, 1NM-PP1-induced CENP-A assembly occurs in a time frame that may allow for complete assembly in these cells. Strikingly, we found that when inhibition of CENP-A assembly is maintained by Cdk1 alone (in the absence of Cdk2), only partial inhibition of Cdk1 is sufficient to induce premitotic CENP-A assembly. This indicates that high Cdk1 activity is required to prevent CENP-A assembly. In conditions where Cdk1as was completely inhibited we observed that SNAP-CENP-A was slightly overloaded at G2 centromeres compared with G1 control cells (Figure 3.13 C).

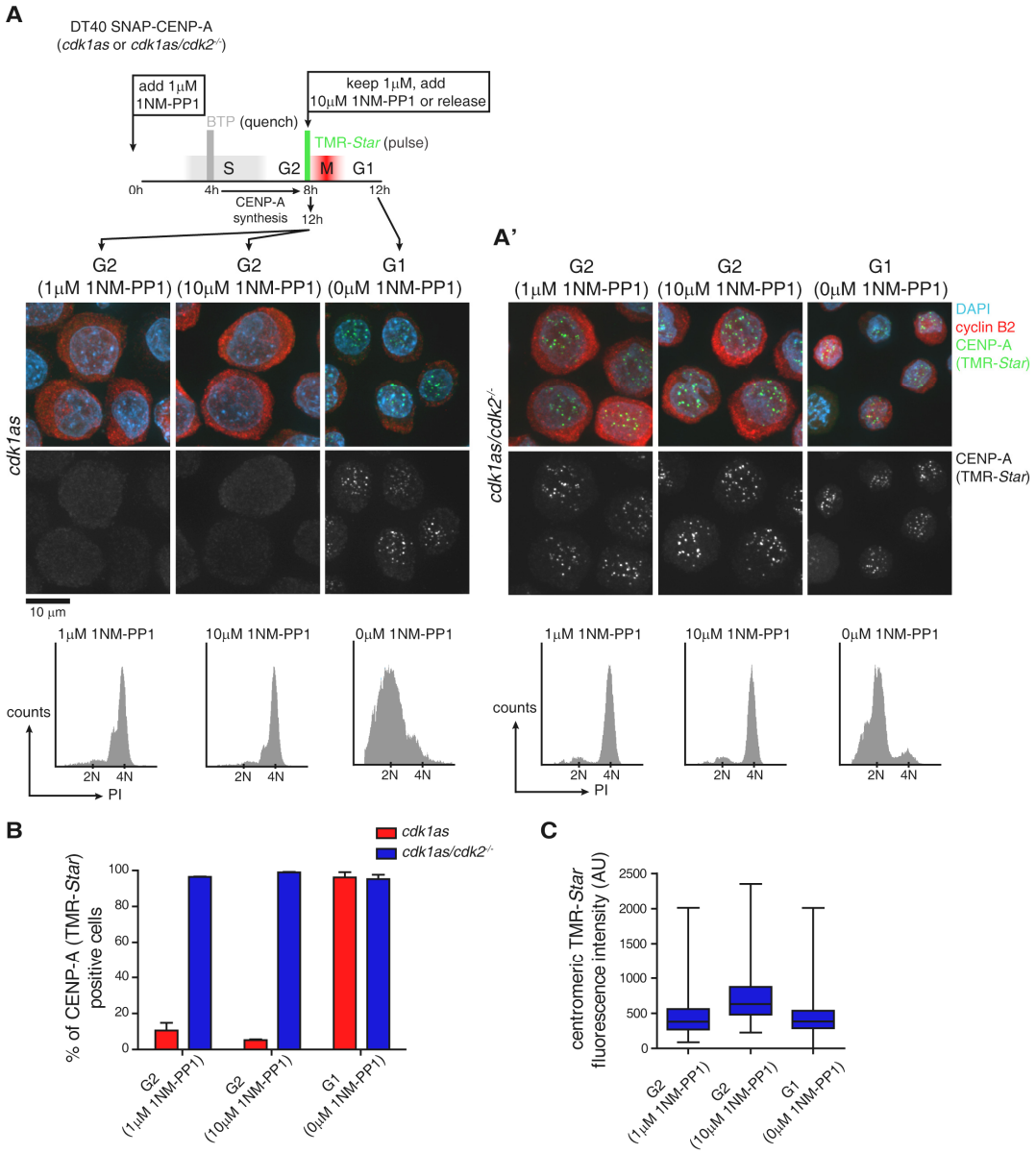

Figure 3.13. CENP-A assembly is suppressed by Cdk1 and Cdk2 activity. (A) DT40 *cdk1as* cells were synchronized in G2 phase as in Figure 3.11 A, followed by TMR-*Star* pulse labelling of a newly synthesized pool of SNAP-CENP-A. Cells were then either kept arrested in G2 with low (1μM) or high (10μM) concentrations of 1NM-PP1 or released into G1 followed by fixation and processing for imaging or flow cytometry. For flow cytometry cells were labelled with PI to stain the DNA. For imaging cells were counterstained for cyclin B2 and with DAPI to indicate G2 status and DNA, respectively. Continued on the next page.

Figure 3.13. (continued) (A´) as A but for *cdk1as/cdk2*$^{-/-}$ double mutant cells. (B) Quantification of A and A´. Mean and SEM of 3 replicates of each condition are shown. ~ 150 cells were analyzed for each replicate. (C) Box and whisker plots of relative CENP-A TMR-*Star* fluorescent signal per centromere in G2 *cdk1as/cdk2*$^{-/-}$ cells treated with low (1 µM) or high (10 µM) levels of 1NM-PP1 and in G1 *cdk1as/cdk2*$^{-/-}$ control cells. CENP-T was used as a reference for centromere position. More than 1000 centromeres were quantified in each condition.

In summary, loss of both Cdk1 and Cdk2 activities is necessary and sufficient to trigger premature CENP-A loading, which indicates that these two kinases are responsible for suppressing the CENP-A assembly machinery prior to mitotic exit.

CENP-A assembly can be induced during S phase in DT40 cells

We next determined whether CENP-A assembly in DT40 cells, induced by the loss of Cdk1 and Cdk2 activities, was restricted to G2 phase as was observed in HeLa cells. To test this, we first inhibited Cdk1 in a randomly cycling population of *cdk1as/cdk2*$^{-/-}$ double mutant cells, which resulted in CENP-A assembly in ~78% of the cells (Figure 3.14 A-C). This result suggests that CENP-A loading can be induced also in S phase, as randomly cycling DT40 cells spend 60% of their time in S phase (Zhao et al., 2007).

To directly test whether Cdk1 and Cdk2 inhibition is sufficient to trigger centromeric chromatin assembly in S phase, we synchronized both *cdk1as* single and *cdk1as/cdk2*$^{-/-}$ double mutant cells in this phase of the cell cycle with Hydroxyurea (HU) and assayed SNAP-CENP-A incorporation at centromeres. In *cdk1as* single mutant cells little or no SNAP-CENP-A assembly was observed, even after complete Cdk1as inhibition (Figure 3.15 A and B), indicating that Cdk2 activity is sufficient to block this process during S phase. However, in *cdk1as/cdk2*$^{-/-}$ double mutant cells ~37% of the cells with active Cdk1 (no

1NM-PP1) assembled SNAP-CENP-A at the centromeres. The percentage of SNAP-CENP-A-TMR-*Star* positive cells increased to ~80% when Cdk1 was further inhibited (10 µM 1NM-PP1) (Figure 3.15 A and B), demonstrating that Cdk1 can contribute, but is not sufficient to prevent CENP-A loading in S phase. These results show that DT40 cells arrested in S phase are competent for CENP-A assembly, and that assembly in S phase is mainly prevented by Cdk2.

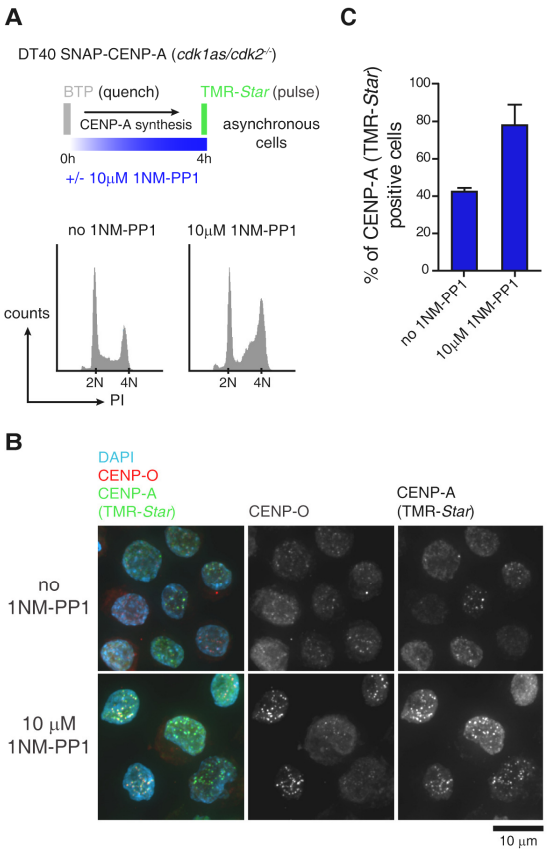

Figure 3.14. Unscheduled CENP-A assembly is not restricted to G2 phase in DT40 cells. (A) Asynchronous cultures of DT40 *cdk1as/cdk2*$^{-/-}$ SNAP-CENP-A cells were treated for 4 hours with DMSO or 10µM 1NM-PP1 as a control or to induce G2 arrest, respectively. During the treatment, a nascent pool of SNAP-CENP-A was synthesized and pulse labeled. Cells were then assayed for cell cycle position by flow cytometry (DNA was stained with PI). (B) Cells were counterstained for CENP-O and with DAPI to indicate centromeres and DNA, respectively, and assayed for CENP-A assembly by microscopy. (C) Quantification of B. Number of cells assembling CENP-A at centromeres was determined. Mean and SEM of 3 replicates of each condition are shown. ~ 100 cells were analyzed for each replicate.

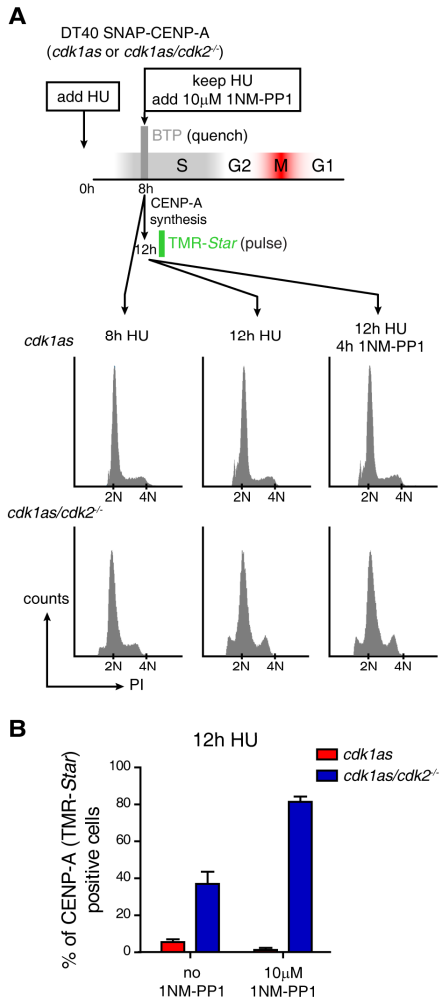

Figure 3.15. S phase arrested DT40 cells are competent for CENP-A assembly. (A) DT40 *cdk1as* and *cdk1as/cdk2*$^{-/-}$ SNAP-CENP-A cells were arrested in S phase by HU treatment. A nascent SNAP-CENP-A pool was labeled in the presence or absence of 10 μM of 1NM-PP1 under continued HU arrest and scored for centromere assembly. Samples were collected for flow cytometry analysis before (8h) and after (12h) nascent SNAP-CENP-A synthesis to monitor continued S phase arrest. Cells were then labelled with PI to stain the DNA and assayed for cell cycle position by flow cytometry (B). Cells were analysed by microscopy as in Figure 3.14 B to assess CENP-A assembly. Number of cells assembling CENP-A at centromeres was determined. Mean and SEM of 3 replicates of each condition are shown. ~ 100 cells were analyzed for each replicate.

HU-arrested cells are not actively replicating DNA as HU treatment reduces the dNTP pool in the cells and, as a result, stalls DNA polymerase at the replication forks (Tyrsted, 1982; Snyder, 1984). To test whether CENP-A loading can occur in cells that are progressing through S phase, we synchronized *cdk1as/cdk2*$^{-/-}$ double mutant cells in early S phase by HU treatment and then released them in the presence of BrdU to mark actively replicating cells. Subsequently, we labeled a nascent SNAP-CENP-A pool, which was synthesized during progression through S phase in the presence or absence of high levels of 1NM-PP1 (10 μM). Following HU release, cells were analyzed by microscopy and

flow cytometry to score for CENP-A assembly and cell cycle position, respectively (Figure 3.16 A and B). In conditions where 70% of the cells are in S phase (BrdU positive) and Cdk1 and Cdk2 were both inhibited, 85% of the cells assembled CENP-A at centromeres (Figure 3.16 A and B). These results demonstrate that DT40 cells undergoing DNA replication are competent for CENP-A assembly.

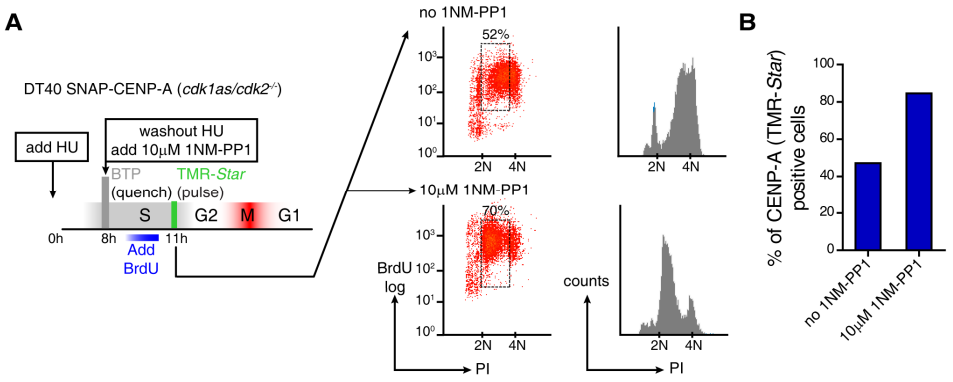

Figure 3.16. Actively replicating DT40 cells are able to assemble CENP-A at centromeres after Cdk1 and Cdk2 inhibition. A) DT40 *cdk1as/cdk2*$^{-/-}$ SNAP-CENP-A cells were arrested in S phase by HU treatment. Cells were subsequently released from HU induced S phase arrest in the presence of BrdU to monitor active DNA replication and in the presence or absence of 10 μM 1NM-PP1. During this time, a new SNAP-CENP-A pool was synthesized. Cells were stained with anti-BrdU antibody and with PI to visualize the S phase status and DNA, respectively. Cells were next analyzed by flow cytometry to determine cell cycle position and S phase progression. Fraction of cells undergoing DNA replication is indicated (boxed region). (B) Cells were analysed by microscopy as in Figure 3.14 B to assess CENP-A assembly. Number of cells assembling CENP-A and actively replicating their DNA was determined in the presence or absence of 10 μM 1NM-PP1. Percentage of cells assembling CENP-A is indicated. ~ 100 cells were analyzed.

Discussion and Conclusions

In this chapter we established that CENP-A displays unique cell cycle dynamics compared with all other centromere proteins analyzed thus far [Figure 3.2 and (Jansen et al., 2007; Hemmerich et al., 2008; Prendergast et al., 2011)]. CENP-A is an extremely stable protein and is quantitatively recycled onto each sister chromatids during DNA replication (Jansen et al., 2007; Dunleavy et al., 2011). The assembly of nascent CENP-A into centromeric nucleosomes is uncoupled from DNA replication and, at least in metazoans, is restricted to late mitosis/early G1 phase of the cell cycle (Jansen et al., 2007; Schuh et al., 2007; Hemmerich et al., 2008; Bernad et al., 2011; Moree et al., 2011). As CENP-A assembly depends on passage through mitosis, it was initially proposed that one or more mitotic events may be driving the activation of this process (Chapter 1, Section 3.2.2). Here we showed that the only aspect of mitosis required for CENP-A incorporation into centromeric chromatin is the concomitant downregulation of Cdk1 and Cdk2 activities upon mitotic exit (Figure 3.3 – 3.6 and 3.9 – 3.13). Moreover, our results demonstrated that the CENP-A assembly machinery is present and poised for activity throughout most of the cell cycle, but is kept in an inactive state until DNA replication and chromosome segregation are completed.

The CCAN proteins can be divided in distinct classes based on their dynamic localization and time of assembly

Using SNAP-based pulse labelling experiments we showed that CENP-N targeting to centromeres, unlike CENP-A assembly, is not limited to early G1 phase of the cell cycle. Instead this CCAN protein is assembled at centromeres throughout the cell cycle, except during metaphase (Figure 3.2). Although this protein displays a dynamic localization at centromeres, a recent study showed that CENP-N becomes stably associated with centromeres during mid S phase

(Hellwig et al., 2011). This shows that even though CENP-N binds directly to CENP-A nucleosomes *in vitro* (Carroll et al., 2009), its centromeric levels rise in S phase, during which pre-existing CENP-A nucleosomes are diluted and redistributed between the two sister chromatids with no new incorporation of this histone. This observation suggests that CENP-N may have an important role in stabilizing CENP-A nucleosomes during redistribution at the DNA replication fork, preventing the loss of the epigenetic mark essential for centromere function and propagation. CENP-I is assembled exclusively during S phase and, as CENP-A, is an extremely stable protein. These characteristics of CENP-I suggest that this protein, like CENP-N, may be involved in stabilizing CENP-A nucleosomes during DNA replication. Indeed, CENP-I depends on CENP-N for its centromere localization (Carroll et al., 2009), which is consistent with its recruitment to the centromeres when CENP-N reaches its maximum centromeric levels. Interestingly, CENP-C and CENP-H are also stabilized at the centromeres during S phase, suggesting that they may act together with CENP-N and CENP-I to maintain CENP-A levels and centromere organization during S phase.

Importantly, most of centromeric proteins tested so far are extremely stable during mitosis, confirming they have an important role in kinetochore formation. CENP-H and CENP-B display dynamic exchange throughout most of the cell cycle, with a stable pool at centromeres during mitosis and S phase or mitosis and G2 phase, respectively (Hemmerich et al., 2008). On the other hand, CENP-T and CENP-W are very stable during G1 and early S phase and are assembled at the centromere after initiation of DNA replication and before mitosis (Prendergast et al., 2011), indicating that these proteins may be involved in changing the centromeric chromatin to a kinetochore-competent configuration. Therefore, the stable centromere core includes distinct classes of proteins, based on their timing and mechanism of assembly, and its composition varies throughout the cell cycle. These distinct characteristics of the CCAN proteins may be related with their specific functions at the centromere.

APC/C-mediated loss of Cdk1 and Cdk2 activities is the unique mitotic feature required for CENP-A assembly

The timing of CENP-A assembly coincides with the completion of the main function of the centromere, which consists in kinetochore assembly, microtubule attachment, mitotic checkpoint function and chromosome segregation. This has raised the possibility that centromere function itself may constitute a signal to initiate CENP-A assembly (Mellone and Allshire, 2003; Carroll and Straight, 2006). However, previous efforts revealed that, in the absence of an active mitotic checkpoint, microtubule attachment, chromosome segregation and proper cytokinesis are not required to ensure proper CENP-A assembly during G1 phase (Jansen et al., 2007; Schuh et al., 2007). Here we confirmed that microtubule attachment and chromosome segregation are not required for CENP-A assembly, even in the presence of mitotic checkpoint proteins (Figure 3.3). Given that none of these processes is required for CENP-A assembly, we conclude that propagation of centromeres is probably not directly triggered by their mitotic functions.

Here, we showed that inhibition of Cdk activity by Roscovitine induced CENP-A loading prior to mitosis (Figure 3.4 and 3.6). Subsequently, using chemical genetics in DT40 cells, we confirmed that Cdk1 and Cdk2 activities are required and sufficient to maintain cell cycle control of CENP-A assembly (Figure 3.11 – 3.13). However, we cannot exclude the possibility that other aspects of mitosis fine-tune CENP-A homeostasis. Indeed, a speculative hypothesis has been proposed where the efficiency of centromere function during metaphase negatively regulates the amount of CENP-A assembled at centromeres upon mitotic exit, forming a negative feedback loop that ensures the maintenance of proper CENP-A levels within strict boundaries (Brown and Xu, 2009). Our results are not inconsistent with the existence of such an additional layer of regulation.

Importantly, we also showed that unscheduled CENP-A assembly, induced by loss of Cdk activity, does not depend on APC/C activation or protein synthesis

(Figure 3.9). This demonstrates that *de novo* synthesis of an assembly factor or APC/C-mediated destruction of a putative inhibitor, other than cyclin A and cyclin B, is not required for CENP-A assembly. Therefore, we conclude that the only mitotic trigger required for stable CENP-A incorporation into centromeric chromatin is the concomitant loss of Cdk activity. Moreover, our results also imply that the CENP-A assembly machinery is present throughout cell cycle but is kept in an inactive state, through Cdk1 and Cdk2 activities, until mitosis is completed.

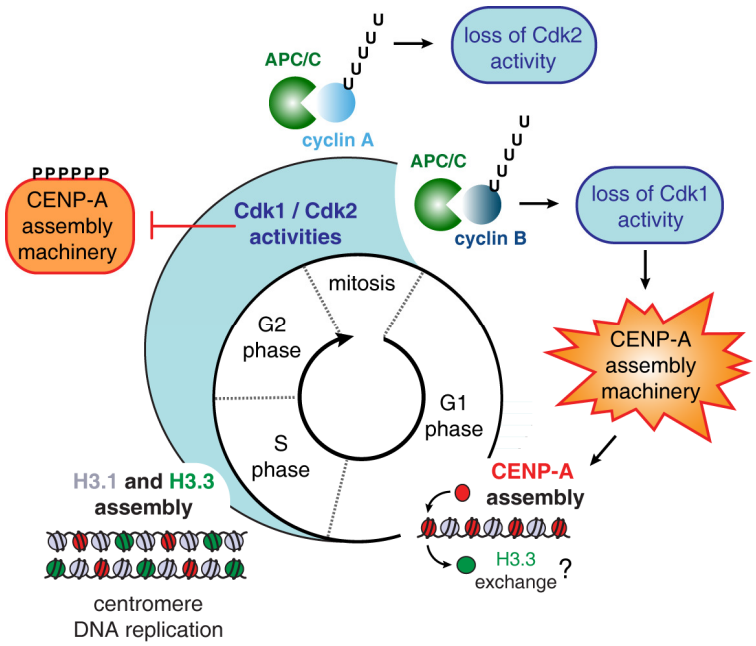

Figure 3.17. Cdk1 and Cdk2 activities maintain cell cycle control of CENP-A assembly. Cartoon illustrating Cdk1/Cdk2 mediated inhibition of CENP-A assembly, exerted perhaps through phosphorylation (P) of one or more CENP-A assembly factors, keeping the CENP-A assembly machinery inactive during S, G2 and M phases. Inhibition is alleviated trough APC/C^{Cdc20} mediated loss of Cdk1 activity in anaphase, leading to dephosphorylation and activation of CENP-A assembly factors. CENP-A assembly in G1 phase possibly involves an exchange with H3.3 (Dunleavy et al., 2011). Canonical (H3 containing) nucleosomes are shown in light blue, H3.3 “placeholder” nucleosomes in green and CENP-A nucleosomes in red.

Here, we propose a model in which Cdk1 and Cdk2 inhibit the CENP-A assembly machinery during most of the cell cycle, possibly through direct phosphorylation of one or more CENP-A assembly factors. Our Cdk1as titration experiments in chicken DT40 cells (Figure 3.13 A') showed that, in the absence of Cdk2, partial Cdk1 activity is not sufficient to maintain inhibition of CENP-A assembly in G2 phase. This suggests that in interphase inhibition is maintained primarily by Cdk2 activity. Cdk2 activity is lost after entry into mitosis through APC/C^{Cdc20}-mediated degradation of its activator cyclin A. This indicates that during prometaphase and metaphase high mitotic Cdk1 activity is sufficient to prevent assembly of CENP-A. Ultimately, the loss of Cdk1 activity mediated by APC/C^{Cdc20}-dependent degradation of cyclin B, during mitotic exit, alleviates the inhibition and allows CENP-A loading to occur (Figure 3.17).

DNA replication and centromere propagation: small molecule inhibitors versus chemical genetics

The results described in this chapter emphasize the advantages of using a chemical genetic approach instead of conventional small molecule inhibitors, which display broad substrate specificity and can induce toxicity and cell death. Using the small molecule inhibitor Roscovitine we showed that Cdk1 and Cdk2 inhibition was not enough to induce CENP-A loading at human centromeres concurrently with DNA replication (Figure 3.7 and 3.8). In contrast, using chemical genetics combined with gene deletion to specifically inhibit Cdk1 and Cdk2, respectively, we demonstrated that inhibition of these two kinases was sufficient to trigger CENP-A assembly during S phase in chicken DT40 cells (Figure 3.14 – 3.16). Although it is possible that CENP-A assembly is controlled differently in human versus chicken cells, we hypothesize that our initial observations in human cells resulted from off-target effects of Roscovitine.

Roscovitine has a broad substrate specificity and strongly inhibits Cdk1 and Cdk2 which are important for cell cycle progression, Cdk5 which is involved in

brain specific processes, and Cdk7 and Cdk9 that are involved in transcription activation (Bach et al., 2005). Cdk7 and Cdk9 activate mRNA transcription through phosphorylation of RNA polymerase II (Fisher, 2005; Marshall and Grana, 2006). Interestingly, CENP-A containing chromatin can be transcribed and neocentromeres can be maintained on active protein-coding genes (Saffery et al., 2003; Nakashima et al., 2005; Lam et al., 2006). In addition, it was recently shown that transcription of centromeric repeats is important for recruitment of the CENP-A specific chaperone HJURP, and consequently for proper incorporation of CENP-A at centromeres on human artificial chromosomes (Bergmann et al., 2011). The accumulation of these α-satellite transcripts starts in S phase and peaks in G2 phase (Ferri et al., 2009), similar to what was observed for HJURP and CENP-A protein levels (Foltz et al., 2009). It is thus conceivable that Roscovitine inhibits centromeric transcription in a way that is rate limiting for CENP-A assembly during S phase but not during G2 phase. An alternative hypothesis to explain why human S phase cells are resistant to Roscovitine-induced CENP-A assembly is a potential diminishment in the expression of genes that produce components of CENP-A assembly machinery. In G2 phase, when levels of CENP-A and HJURP are high, a Roscovitine mediated repression of transcription of these genes may not be enough to prevent CENP-A assembly, whereas during S phase repression may be rate limiting. Another, and perhaps more attractive, hypothesis is the existence of an activation step during S phase, similar to the one controlling DNA replication. This would invoke a kinase (Cdk1, Cdk2, Cdk4, Cdk5, Cdk7, Cdk9 or an unknown target of Roscovitine) that primes (perhaps through phosphorylation) the CENP-A assembly machinery in S phase, allowing its subsequent activation upon Cdk1 and Cdk2 inhibition. Roscovitine addition to S phase cells that are not yet primed for CENP-A assembly may inhibit the priming kinase preventing CENP-A assembly to occur even after Cdk1 and Cdk2 inhibition.

Using DT40 cells that express an analog-sensitive version of Cdk1 in a homozygous *cdk2* and/or *cdk1* null background, we demonstrated that loss of

both Cdk1 and Cdk2 activities induces unscheduled CENP-A assembly in S and G2 phases. Although Cdk1 activity is sufficient to rescue S phase entry and S phase progression in the absence of Cdk2, it is not enough to completely inhibit CENP-A assembly in S phase (Figure 3.15 B and 3.16 B). This result indicates that Cdk2 has a primary role in preventing CENP-A assembly during this phase of the cell cycle. Cdk2 activity is not essential for DT40 cells and mouse viability (Berthet et al., 2003; Ortega et al., 2003; Hochegger et al., 2007), because Cdk1 can be activated not only by cyclin B, but also by interphase cyclins, such as cyclins D, E and A (Aleem et al., 2005; Santamaría et al., 2007). Conversely, cyclin B can bind to and activate Cdk2 (Brown et al., 2007; Petri et al., 2007) but Cdk2-cyclin B activity is not sufficient to perform all Cdk1 functions and to rescue Cdk1 lethality (Satyanarayana et al., 2008). This shows that although Cdk1 and Cdk2 bind to the same cyclins and inhibitors, they are not fully redundant. Although at the first glance Cdk2 appears to be redundant with Cdk1, recent findings highlight nonredundant roles for Cdk2 in cell cycle progression (Merrick et al., 2008, 2011). Our results now reveal another nonredundant role of Cdk2 in preventing centromere propagation during S phase. Despite the ability of most DT40 cells to assemble CENP-A during S phase, we observed a significant percentage (~ 20%) of S phase cells unable to do so. This observation may indicate the existence of an additional level of control during S phase that cannot be explained by off-target effects of the Cdk1as inhibitor. This may possibly hint at a priming step by an unknown kinase, or by Cdk1 or Cdk2, as it was above described.

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Chapter 4 – Molecular mechanism maintaining cell cycle control of CENP-A assembly

Abstract

Centromeres, the chromosomal loci that form the sites of attachment for spindle microtubules during mitosis, are identified by a unique chromatin structure generated by nucleosomes containing the histone H3 variant CENP-A. Propagation of CENP-A chromatin is uncoupled from DNA replication initiating only during mitotic exit. We previously demonstrated that inhibition of Cdk1 and Cdk2 activities is sufficient to trigger CENP-A assembly throughout the cell cycle. We now reveal that this unscheduled loading of CENP-A into centromeric chromatin depends on the canonical CENP-A assembly machinery. We further show that the key CENP-A assembly factor Mis18BP1^{HsKNL2} is phosphorylated in a cell cycle dependent manner which controls its centromere localization during mitotic exit. These results strongly support a model where the CENP-A assembly machinery is poised for activation throughout the cell cycle but kept in an inactive, noncentromeric state through Cdk activity during S, G2 and M phases. Alleviation of this inhibition in G1 phase ensures tight coupling between DNA replication, cell division, and subsequent centromere maturation.

Introduction

The centromere is a specialized chromosomal locus that drives the assembly of the kinetochore, allowing proper chromosome segregation during mitosis (Allshire and Karpen, 2008; Cheeseman and Desai, 2008). In most eukaryotes, centromeres are propagated epigenetically, largely independent of any particular DNA sequence (Vafa and Sullivan, 1997; Warburton et al., 1997; Warburton, 2004). The centromere protein A (CENP-A), a histone H3 variant exclusively present in centromeric nucleosomes, acts as an epigenetic mark responsible for centromere propagation and function (Silva and Jansen, 2009; Barnhart et al., 2011; Mendiburo et al., 2011). Unlike canonical histones, which are assembled during DNA replication throughout chromatin, the assembly of nascent CENP-A into centromeric chromatin is restricted to early G1 phase of the cell cycle (Bernard et al., 2001; Jansen et al., 2007; Schuh et al., 2007; Hemmerich et al., 2008; Moree et al., 2011).

As described in Chapter 2, formation of mature CENP-A chromatin occurs in three distinct steps: licensing, assembly and stabilization. The two first steps have been extensively characterized and require the function of the Mis18 complex and HJURP, respectively. The licensing step is performed during anaphase by the Mis18 complex, which includes Mis18α, Mis18β and Mis18BP1^{HsKNL2} (Fujita et al., 2007; Maddox et al., 2007; Moree et al., 2011). Centromere targeting of the Mis18 complex precedes and is essential for CENP-A assembly. Mis18 licensed chromatin is then recognized by the specific CENP-A chaperone HJURP during late telophase/early G1 phase (Dunleavy et al., 2009; Foltz et al., 2009; Barnhart et al., 2011). HJURP binds to CENP-A prenucleosomal complexes and acts to deliver and assemble these complexes into centromeric chromatin during late telophase/early G1 (Dunleavy et al., 2009; Foltz et al., 2009; Shuaib et al., 2010).

Assembly of CENP-A and centromere targeting of the CENP-A assembly factors described above depends on passage through mitosis, ensuring a direct

coupling of centromere propagation to cell cycle progression (Fujita et al., 2007; Jansen et al., 2007; Maddox et al., 2007; Schuh et al., 2007; Dunleavy et al., 2009; Foltz et al., 2009). In Chapter 3 we identify the mitotic signal that restricts CENP-A assembly to late mitosis/early G1 phase. We reveal that inhibition of Cdk1 and Cdk2 activity is sufficient to trigger CENP-A assembly throughout the cell cycle. Additionally, we show that neither protein degradation nor protein synthesis is required for CENP-A assembly induced by premature loss of Cdk1 and Cdk2 activities (Chapter 3). Our results suggest that the CENP-A assembly machinery is present throughout cell cycle but is kept in an inactive state by Cdk1 and Cdk2 activities. One possibility is that Cdk1 and Cdk2 inhibit the CENP-A assembly machinery, during most of the cell cycle, through direct phosphorylation of one or more CENP-A assembly factors.

Here, we investigated the molecular mechanism through which Cdk1 and Cdk2 control G1 phase timing of CENP-A assembly. We demonstrate that high Cdk activity prevents centromere targeting of the well characterized CENP-A assembly factors: Mis18α, Mis18BP1^{HsKNL2} and HJURP. Moreover, we show that these CENP-A assembly factors are required for unscheduled CENP-A assembly, indicating that premature loading occurs through the canonical pathway. Importantly, we demonstrate that Mis18BP1^{HsKNL2} is highly phosphorylated during mitosis, which prevents its centromere targeting. We also show that a domain of HJURP, which is conserved among vertebrates, is important to regulate the cell cycle timing of centromere propagation. Taken together, our results lead to a model in which Cdk1 and Cdk2 inhibit CENP-A assembly during most of the cell cycle through phosphorylation of Mis18BP1^{HsKNL2} and potentially other assembly factors.

Material and Methods

Cell lines and constructs

HeLa cells and their derivatives were cultured as described in Chapter 2. In this chapter we used HeLa cell lines stably expressing Mis18α and/or CENP-A-SNAP (Chapter 2). We also used a HeLa cell line stably expressing LAP-(GFP)-HJURP (referred to as GFP-HJURP throughout this chapter; a gift from D. Foltz, University of Virginia, Charlottesville, VA).

LAP-(GFP)-Mis18α and LAP-(GFP)-Mis18β constructs named pLJ383 and pLJ382, respectively, were a gift from I. Cheeseman, MIT, Cambridge, MA. A HJURP-(GFP)-LAP construct named pLJ381 was a gift from D. Foltz, University of Virginia, Charlottesville, VA. A CMV-CENP-A-SNAP-3xHA construct named pLJ511 was generated by inserting a PCR-generated fragment carrying the human CENP-A open reading frame (ORF) fused with SNAP-3xHA and flanked by BglII and NotI sites into the corresponding sites of the Clontech vector pEYFP-C1 (replacing YFP with the CENP-A-SNAP-3xHA ORF). The Mis18BP1^{HsKNL2} ORF was amplified from a cDNA clone (kind gift from Paul Maddox) by PCR and cloned into the XhoI and EcoRI sites of pIC113 [a gift from Iain Cheeseman, MIT, Cambridge, MA; (Cheeseman and Desai, 2005)], creating pLJ415 expressing a LAP-(GFP)-Mis18BP1^{HsKNL2} fusion protein. The 24 Alanine mutant version of Mis18BP1^{HsKNL2} was generated synthetically by GeneArt (pLJ450) and was subcloned into XhoI and EcoRI sites of pIC113 resulting in pLJ451. The construct expressing the Mis18BP1^{HsKNL2}-Ala^{24} mutant (pLJ451) is identical to pLJ415 except that residues S110, S134, S135, S191, S192, T260, T261, S263, S299, S365, S541, T653, T821, S824, S914, S991, T992, T993, S1004, S1008, S1086, S1087, S1089, S1104 are mutated to Alanine.

Site-directed mutagenesis

Mis18α, Mis18β, CENP-A and HJURP phosphomutants were generated by site-directed mutagenesis using the constructs listed above and the primers listed in the Table 4.1. The primers were designed according to the instructions of the QuickChange Site-Directed Mutagenesis Kit.

Table 4.1. List of primers used for site-directed mutagenesis

Mutant protein	Primer name	Primer sequence
GFP-Mis18α^{S233A} (pLJ474)	Mis18a_5EcoRI	GAGAATTCTCGCAGGCGTTCGGTCACTGAG
	3BamHI_Ala-Mis18a	GGTGGATCCTCAGGCTTTACAAGTGGCAAAGGACAATTTGG
GFP-Mis18β^{S47A,S48A} (pLJ457)	Mis18b_S34A-S35A_F	ACGCAGGTGGTGAAGGGGGCCGCCCCGCTCGGCCCCGCAGGG
	Mis18b_S34A-S35A_R	CCCTGCGGGGCCGAGCGGGGCGGCCCCCTTCACCACCTGCGT
GFP-Mis18β^{S221A,S225A} (pLJ458)	Mis18b_T221A-S225A_F	GATTCTGAGTGAAGTGGCTCCTGACCAGGCCAAGCCAGAAAACTG
	Mis18b_T221A-S225A_R	CAGTTTTCTGGCTTGGCCTGGTCAGGAGCCACTTCACTCAGAATC
CENP-A^{S7A}-SNAP (pLJ513)	5-Mut-S7A	CGCAGATCTACCATGGGCCCGCGCCGCCGGGCCCGAAAGCCCGAGGCCC
	CA3-Xho	CGACCTCGAGAAGGCCGAGTCCCTCCTCAAGGCCCC
CENP-A^{S7E}-SNAP (pLJ514)	CA3-Xho	CGCAGATCTACCATGGGCCCGCGCCGCCGGGAGCGAAAGCCCGAGGCCC
	5-Mut-S7E	CGACCTCGAGAAGGCCGAGTCCCTCCTCAAGGCCCC
HJURP^{S412A}-GFP (pLJ412)	CenCAF_5S412A	GACATTAAAATGGTTAATTGCTCCTGTAAAAATAGTTTC
	CenCAF_3S412A	GAAACTATTTTTACAGGAGCAATTAACCATTTTAATGTC

PCR reactions were performed with 12 pmol of each primer, 50 ng of each construct described above, 10 nmol dNTPs, 2,5 U of Pfu polymerase, and Pfu buffer with $MgSO_4$ (Fermentas). Reaction was run for 18 cycles (30'' at 95ºC, 1' at 55ºC and 12' at 68ºC), followed by treatment with DpnI at 37ºC for 1 hour. Dpn1-treated DNA was transformed into an XL1 bacterial strain. We generated Mis18α^{S233A}, CENP-A^{S7A} and CENP-A^{S7E} fragments by a standard PCR reaction with Vent polymerase. PCR fragments, obtained either by standard PCR or by site directed mutagenesis PCR, were cloned into pIC113 resulting in the constructs listed in Table 4.1. Mis18β^{4Ala} (pLJ461) was generated by digesting pLJ458 with ScaI and BamHI, and by cloning the 332 bp fragment into the same sites of pLJ457 (listed in table 4.1 between brackets).

Note that transfections of the phosphomic mutant of CENP-A (CENP-A^{S7E}) in HeLa cells resulted in very few GFP-positive cells, suggesting that this mutant is unstable and perhaps nonfunctional (data not shown).

Strand exchage PCR

To build the deletion mutants listed in Table A2 in Appendix 1 and the HJURP-Ala^{8} mutant we used a method called strand exchange PCR or overlap extension PCR (Figure 4.1). For details about this method see also Shevchuk et al. (2004). The primers used for strand exchange PCR are listed in Table A1 in Appendix 1.

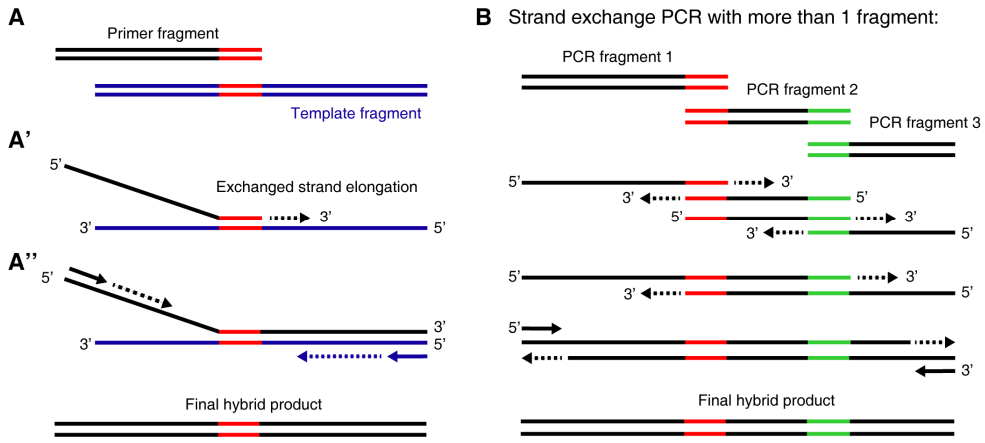

Figure 4.1. Strand exchange PCR method. (A) When fusing two DNA fragments one will serve as a template for primer extension and the other will serve as a primer to be extended. The primer fragment carries homology (red region) to the target template at its 3' end. The template fragment can be a PCR product or an intact plasmid. (A') In the first PCR reaction no oligonucleotides are added. The primer fragment carries a small region of homology that allows it to exchange strands with the template fragment and to be elongated. Continued on the next page. We used 200 fmol of both fragments and Vent polymerase, and performed a PCR reaction of 15 cycles (the annealing temperature was adjusted according with the homology domain between the fragments). (A'') 10 µL of this strand exchange fusion product was used as a template in a second PCR reaction which included oligonucleotides. We used 30 pmol of each primer (represented as black and blue arrows) and Vent polymerase in a PCR reaction of 35 cycles. The final hybrid product was Gel purified and cloned into pIC113 (Cheeseman and Desai, 2005). (B) This method works using more than two primer fragments (either synthesised primers, digestion fragments or PCR products) if each fragment has a unique homology to the next fragment. We have succeeded in obtaining clones this way using up to 6 fragments, covering a total of > 2kb in a single reaction (in the case of the HJURP-Ala^{8}-GFP construct).

Cell synchronization, drug treatments and SNAP quench-chase-pulse labeling

Cells were synchronized as described in Chapter 2. Roscovitine (Sigma) was used at 100 µM. MLN8054 (Selleck Chemicals) and ZM447439 (Enzo Life Sciences) were used at 1 µM and 2 µM, respectively. DMSO (Sigma) was used in control conditions. The quench-chase-pulse labeling was performed as described in Chapter 2.

siRNA and DNA transfections

All siRNAs were obtained from Dharmacon. Smart pools were used to deplete Mis18BP1^{HsKNL2} and HJURP. CENP-A, Mis18α and GAPDH were depleted using single target siRNA oligos. Oligos are listed in Table 2.1 in Chapter 2. The siRNA tranfections were performed as described in Chapter 2.

HeLa cells were transfected with 250 ng of DNA, 1 µl Plus Reagent and 1,25 µl of Lipofectamine (Invitrogen) in Optimem reduced serum media (Gibco) according to manufactures instructions for all Figures except for Figure 4.11 D. For Figure 4.11 D, HeLa cells were transfected with 400 ng of plasmid DNA using Effectene transfection reagent (Qiagen) in Optimem according to the manufacturer's instructions.

Immunoblotting

Extracts of 10^5 HeLa cells were separated in a 6% (Figure 4.11 D) or 12% (Figure 4.4 C) SDS-PAGE gel and transferred to a PVDF membrane. Blots were probed with anti-HJURP and anti-GFP [both a gift from D. Foltz, University of Virginia, Charlottesville, VA; (Foltz et al., 2009)] at 1:2000 and 1:10.000 dilution, respectively. For blot shown in Figure 4.10 D, anti-GFP was used at 1:1000 dilution overnight at 4°C. Anti-Mis18BP1^{HsKNL2} antibody (A302-824A, Bethyl Labs) was used at 1:5000 dilution overnight at 4°C. Anti-mouse and anti-rabbit HRP-

conjugated secondary antibodies were purchased from Jackson Immunoresearch Laboratories.

Phosphatase Treatment

Lysates were prepared from a HeLa cell line stably expressing LAP-(GFP)-Mis18BP1^{HsKNL2} that had been blocked for 12 hours with 100 ng/ml Nocodazole. Cells enriched in mitosis were harvested with 3 mM EDTA-PBS for 10 minutes at room temperature (RT). Cells were resuspended in buffer containing 75 mM HEPES (pH 7.5), 1.5 mM EGTA, 1.5 mM $MgCl_2$, 150 mM KCl, 15% glycerol, 0.075% IGEPAL, 10mM Imidazole (Sigma), 200 mM Sodium Orthovanadate (NaV; MP Biomedicals), 5 mM sodium fluoride, 50 mM β-glycerophosphate, and Complete EDTA-free Protease Cocktail (Roche) and sonicated on ice for 30 second cycles for a total of 2 minutes. Lysates were centrifuged for 10 minutes at 1000 x g at 4ºC and the supernatant was passed five times over a column containing His-tagged GFP binding protein (GBP) bound to Ni-NTA agarose. Proteins bound to the GBP beads were washed, resuspended in 30 mL wash buffer and incubated with 10 mM NaV and/or 60 units of Calf Intestinal Alkaline Phosphatase (CIP; New England Biolabs) for 1 hour at 37ºC. Reactions were stopped by the addition of SDS sample buffer and separated by SDS-PAGE.

Immunofluorescence

HeLa cells were grown on glass coverslips [thickness: 1.5, coated with poly-L-Lysine (Sigma)]. For Mis18BP1^{HsKNL2} immunostaining, cells were pre-extracted with 0,1% triton for 3 minutes and fixed in MetOH (pre cold) at -20ºC during 20 minutes. After fixation cells were washed twice with TBS-0,1%-Triton for 5 minutes and once with RSB buffer (10 mM Tris-HCl [pH 7.4], 210 mM NaCl, and 5 mM $MgCl_2$) for 20min.

For other stainings, HeLa cells were fixed with 4% formaldehyde for 10 min, followed by extraction with 0,1 % Triton X-100 for 5 minutes, and processed for immunofluorescence using standard procedures. Cells were stained with anti-cyclin B1 (sc-245, Santa Cruz) at 1:50 dilution, anti-CENP-T [gift from D. Foltz, University of Virginia, Charlottesville, VA; (Barnhart et al., 2011)] at 1:1000 dilution, anti-Mis18BP1^{HsKNL2} (gift from P. Maddox, Université de Montréal, Montreal, Canada) at the concentration of 1 µg/mL, and anti-HA (clone HA11, Covance) at the concentration of 1 µg/mL. Secondary antibodies (FITC- or Cy5-conjugated anti-mouse, FITC- or Cy5-conjugated anti-rabbit and FITC-conjugated anti-rat) were obtained from Jackson Immunoresearch Laboratories. Dy680 conjugated anti-mouse antibodies were from Rockland Immunochemicals. Cells were stained with DAPI (4',6-diamidino-2-phenylindole; Sigma) before mounting in Mowiol.

Microscopy and fluorescence quantification

Widefield fluorescence microscopy was performed using a DeltaVision Core system (Applied Precision) as described in Chapter 2. All images presented are maximum intensity projections of deconvolved pictures. Centromeric TMR-*Star* fluorescence intensity was quantified as described in Chapter 2.

Results

Cdk inhibition results in unscheduled recruitment of CENP-A assembly factors to centromeres

The Mis18 complex, which includes Mis18α, Mis18β and Mis18BP1^{HsKNL2}, is recruited to the centromere during anaphase just prior to CENP-A and is essential for assembly of this histone variant in late telophase/early G1 phase [Figure 2.1 and 2.2 in Chapter 2 and (Fujita et al., 2007; Maddox et al., 2007)]. The localization of HJURP, another CENP-A assembly factor, is also temporally controlled (Dunleavy et al., 2009; Foltz et al., 2009). This CENP-A specific chaperone is part of the CENP-A/H4 prenucleosomal complex and is targeted to centromeres upon mitotic exit along with CENP-A (Dunleavy et al., 2009; Foltz et al., 2009). CENP-A assembly can be induced prior to mitosis when Cdk1 and Cdk2 are inhibited without the need for new protein synthesis (Figure 3.9 A, B and D in Chapter 3), which indicates that CENP-A assembly factors are present throughout the cell cycle but are kept in an inactive state through Cdk1 and Cdk2 activities. Thus, we hypothesised that Cdk activity may also control the localization of the proteins required for CENP-A assembly. To test this hypothesis, we employed a previously established double tagged HeLa cell line expressing CENP-A-SNAP and GFP-Mis18α (Chapter 2). We showed that inhibition of Cdk activity in G2 phase results in rapid recruitment of GFP-Mis18α and CENP-A-SNAP assembly at centromeres (Figure 4.2). Cells displayed either centromere localized GFP-Mis18α alone (Figure 4.2 A, red arrow) or both GFP-Mis18α and nascent CENP-A-SNAP (Figure 4.2 A, green arrow) but never CENP-A-SNAP alone (Figure 4.2 B). This shows that, as for canonical G1 loading of CENP-A, Mis18α arrives at the centromere prior to CENP-A assembly in G2 phase under Roscovitine-induced conditions.

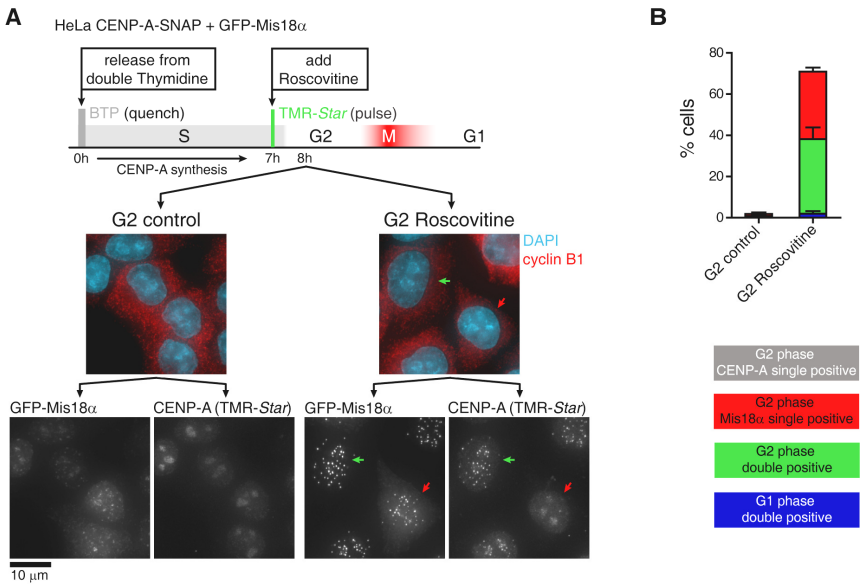

Figure 4.2. Roscovitine treatment induces premature targeting of GFP-Mis18α to centromeres followed by CENP-A assembly in G2 phase. (A) CENP-A-SNAP, GFP-Mis18α double tagged HeLa cells were synchronized at the G1/S boundary by double Thymidine arrest. S phase synthesized CENP-A-SNAP was subsequently pulse labeled in G2 phase, 7 hours after release from Thymidine. G2 cells were either mock treated (G2 control) or treated with Roscovitine for 1 hour (G2 Roscovitine) prior to fixation. Cells were counterstained for cyclin B and with DAPI to indicate G2 status and DNA, respectively. Cells were imaged to determine GFP-Mis18α and nascent CENP-SNAP centromere localization. (B) Quantification of A. Mean and standard error of the mean (SEM) of 3 replicates are shown. ~200 cells were analyzed in each replicate.

Additionally, we demonstrated that endogenous Mis18BP1^{HsKNL2} and stably expressed GFP-HJURP were also rapidly targeted to centromeres prior to mitosis upon Cdk inhibition (Figure 4.3). Together these results strongly suggest that G2 phase-induced CENP-A assembly occurs through its canonical assembly pathway, requiring centromere targeting of the Mis18 complex and HJURP.

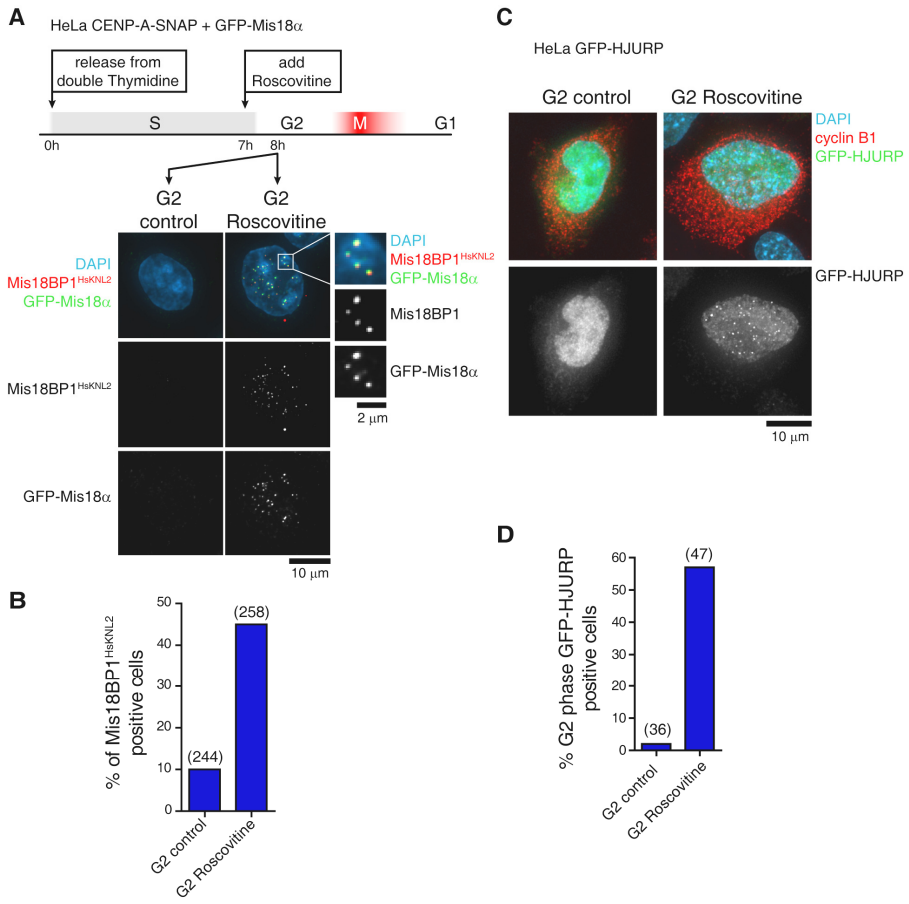

Figure 4.3. Cdk inhibition induces rapid recruitment of Mis18BP1^{HsKNL2} and GFP-HJURP to centromeres in G2 phase. (A) HeLa cells stably expressing CENP-A-SNAP and GFP-Mis18α were treated as in Figure 4.2 A. G2 control or Roscovitine treated cells were counterstained for Mis18BP1^{HsKNL2} and DAPI, and imaged to determine GFP-Mis18α and Mis18BP1^{HsKNL2} centromere localization. (B) Quantification of A. Number of cells analyzed is indicated between brackets. (C) Randomly cycling HeLa cells stably expressing GFP-HJURP were either mock treated or treated with Roscovitine for 1 hour prior to fixation. Cells were counterstained for cyclin B and with DAPI to indicate G2 status and DNA, respectively. Cells were imaged to determine GFP-HJURP centromere localization. (D) Quantification of C. Number of cyclin B positive cells analyzed is indicated between brackets.

Unscheduled CENP-A assembly requires the canonical CENP-A assembly factors

Above, we showed that CENP-A assembly factors relocalize to the centromere in response to Cdk inhibition in G2 phase. Thus, we hypothesized that these factors are required for consequent CENP-A assembly in G2 phase. To test this prediction directly, we used the assay we developed in Chapter 2 (Figure 2.1, Chapter 2). We depleted Mis18α, Mis18BP1^{HsKNL2} or HJURP by RNA interference and measured the amount of nascent CENP-A-SNAP loaded at centromeres in G2 Roscovitine treated cells and in G1 cells.

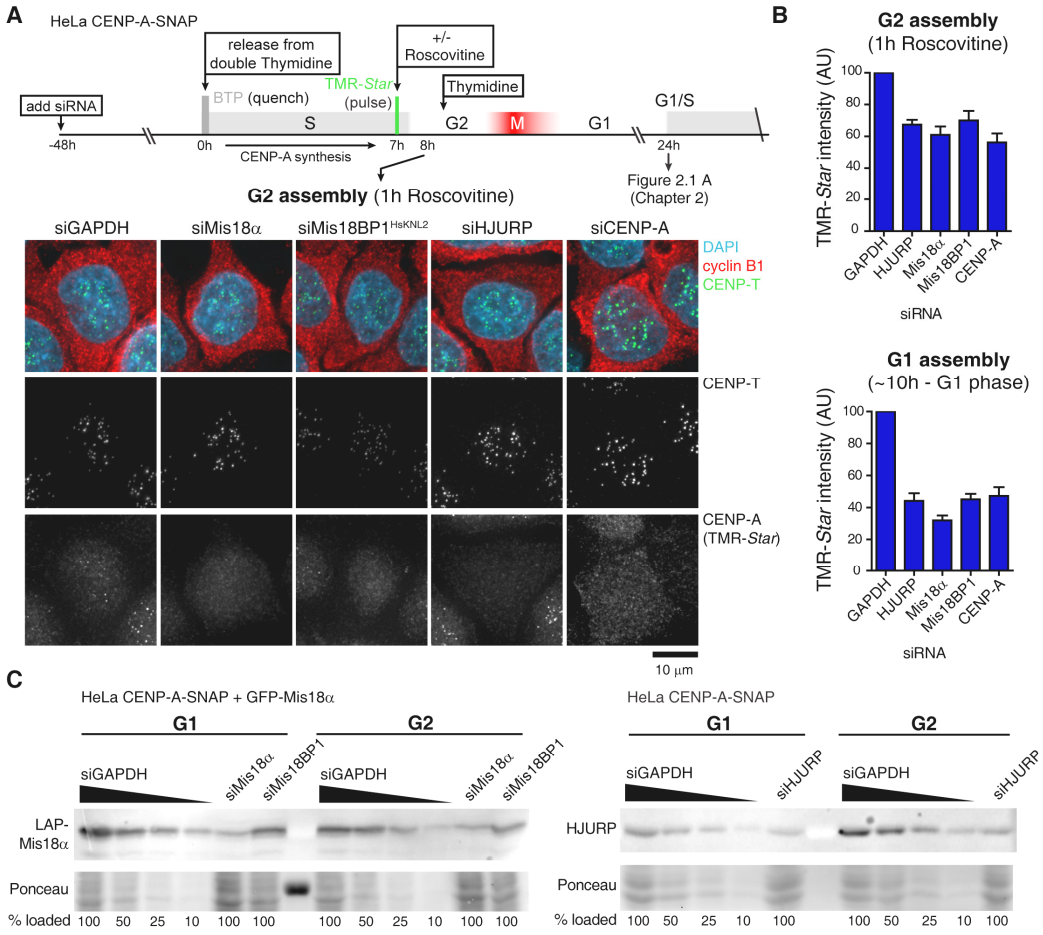

Figure 4.4. Roscovitine-induced CENP-A assembly requires the Mis18 complex and HJURP. Continued on the next page.

Figure 4.4. (continued) (A) HeLa CENP-A-SNAP cells were treated with siRNAs against indicated targets (GAPDH and CENP-A serve as negative and positive controls, respectively) and synchronized by double Thymidine block combined with SNAP quench-chase-pulse labeling. Cells were treated with Roscovitine for 1 hour in G2 to induce CENP-A assembly or were cycled into the next cell cycle and collected at the next G1/S boundary following canonical CENP-A assembly. Cells were imaged and counterstained for CENP-T, cyclin B and with DAPI to indicate centromeres, G2 status and DNA respectively. (B) TMR-*Star* intensity was quantified in more than 1200 centromeres per condition. Mean and SEM of 3 replicates of each condition are shown. (C) HeLa cells expressing CENP-A-SNAP or expressing both CENP-A-SNAP and GFP-Mis18α were transfected with siRNAs and synchronized as in A, followed by processing for SDS-PAGE and immunoblotting. Fraction of cells loaded is indicated for each condition. Efficiency of depletion of Mis18α and Mis18BP1^{HsKNL2} is assessed by GFP-Mis18α protein levels using anti-GFP antibodies [HsMis18α and Mis18BP1^{HsKNL2} are interdependent (Fujita et al., 2007)]. Efficiency of depletion of HJURP is determined using antibodies against endogenous HJURP. Ponceau staining was used as a loading control.

Depletion of these CENP-A assembly factors resulted in a reduction of both G1 phase assembly of nascent CENP-A as well as unscheduled, Roscovitine induced assembly in G2 phase (Figure 4.4 A and B, see also Figure 2.1 in Chapter 2). Immunoblot analysis of siRNA treated cells revealed a modest depletion of assembly factors (Figure 4.4 C). Yet this reduction in Mis18α, Mis18BP1^{HsKNL2} and HJURP was sufficient to cause a defect in CENP-A assembly in both phases. This shows that these proteins are required and rate limiting for both canonical as well as G2 induced CENP-A assembly.

Canonical CENP-A assembly in G1 and Roscovitine-induced assembly in G2 do not require Aurora A and Aurora B activities

Our results thus far show that CENP-A assembly is controlled by two main cell cycle control kinases, Cdk1 and Cdk2. In Chapter 3 we proposed a model in which the CENP-A assembly machinery is inhibited during most of the cell cycle through direct Cdk1 and Cdk2 phosphorylation of one or more CENP-A assembly factors (Figure 3.17 in Chapter 3). However, it remains unclear whether Cdk1 and Cdk2 act in a direct manner or indirectly through a signaling cascade that activates downstream kinases. Aurora A and Aurora B are two well known kinases acting downstream of Cdk and play distinct functions during mitosis. Aurora A regulates entry into mitosis, centrosome maturation and spindle assembly, while Aurora B ensures accurate chromosome segregation, sister chromatid and centromeric cohesion, and proper execution of cytokinesis (Carmena et al., 2009). Aurora A and Aurora B phosphorylate CENP-A during mitosis (Zeitlin et al., 2001; Kunitoku et al., 2003). Together, these observations make Aurora A and Aurora B strong candidates of being the direct targets of Cdk, responsible for the maintenance of cell cycle control of CENP-A assembly. To test this hypothesis we treated HeLa cells stably expressing CENP-A-SNAP with specific inhibitors of either Aurora A (MLN8054) or Aurora B (ZM447439) (Ditchfield et al., 2003; Hoar et al., 2007). We observed that cells treated with these inhibitors loaded CENP-A-SNAP at centromeres exclusively during G1 phase and at levels comparable to control conditions (Figure 4.5 A). Moreover, inhibition of Aurora A and Aurora B activities did not significantly block Roscovitine-induced CENP-A assembly in G2 phase (Figure 4.5 B). Nevertheless, the efficiency of these inhibitors was evident from chromosome segregation defects in mitosis after Aurora A inhibition (asterisk in Figure 4.5 A) or cytokinetic failure and consequent multinucleated cells resulting from Aurora B inhibition (asterisk in Figure 4.5 A). Together, these observations suggest that Aurora A and Aurora B activities are not required for CENP-A incorporation at

centromeres and are not part of a signaling cascade controlling the timing of CENP-A assembly.

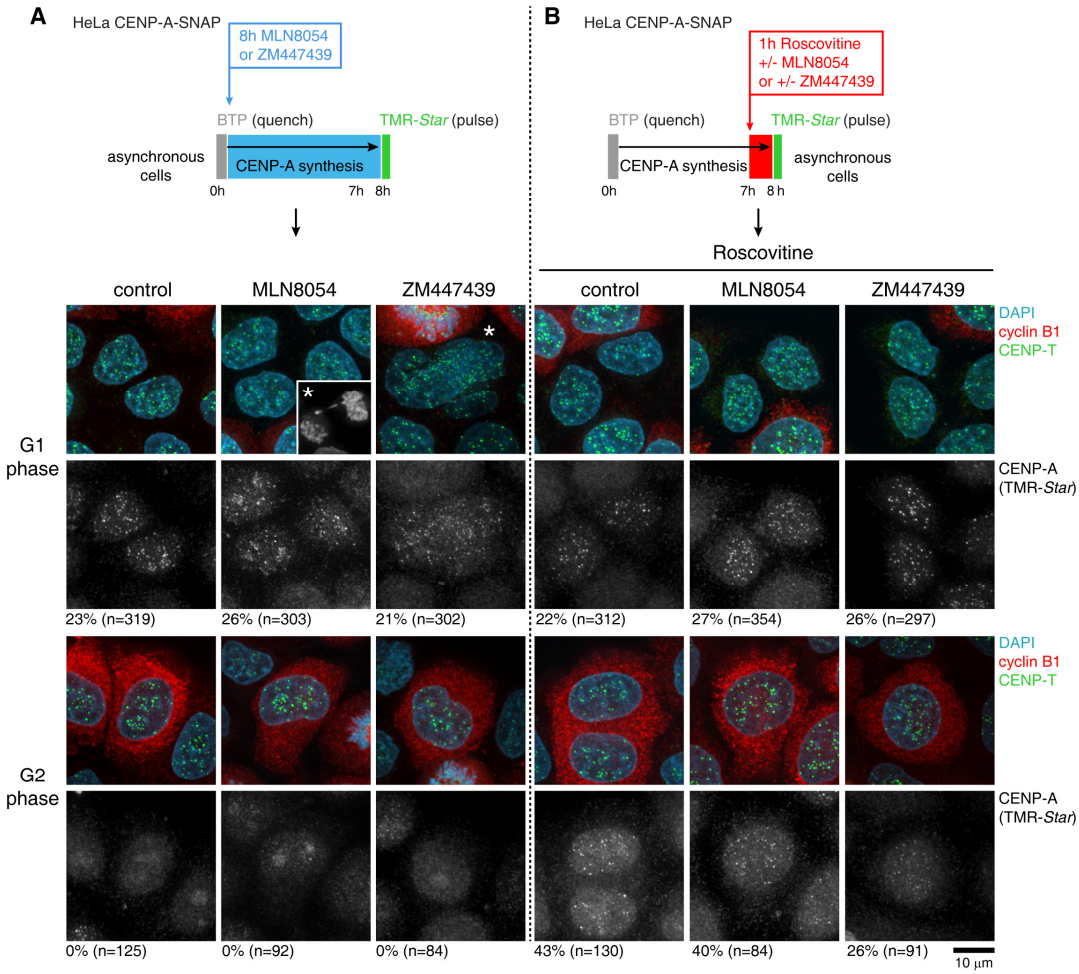

Figure 4.5. Aurora A and Aurora B activities do not influence the timing of CENP-A assembly. (A) Randomly cycling HeLa cells stably expressing CENP-A-SNAP were quench-chase-pulse labeled. During the chase period, either Aurora A or Aurora B activity was inhibited by treatment with 1µM of MLN8054 or 2µM of ZM447439, respectively, followed by fixation and staining for indicated markers. Representative images of cells in G1 (low cyclin B) and G2 phase (high cyclin B) are shown. (B) Experiment as in A except that cells were treated for one hour with either Roscovitine alone or in combination with MLN8054 or ZM447439 prior to fixation. Cells were imaged and counterstained for CENP-T, cyclin B and with DAPI to indicate centromeres, G2 status, and DNA, respectively. Percentage of cells assembling CENP-A-SNAP and the number of cells analyzed (n) is indicated.

Consistent with these results, conversion of the known Aurora A/Aurora B phosphorylation site Serine 7 (Zeitlin et al., 2001; Kunitoku et al., 2003) to Alanine in CENP-A did not block its assembly at centromeres nor did it alter the G1 phase timing of its assembly (Figure 4.6).

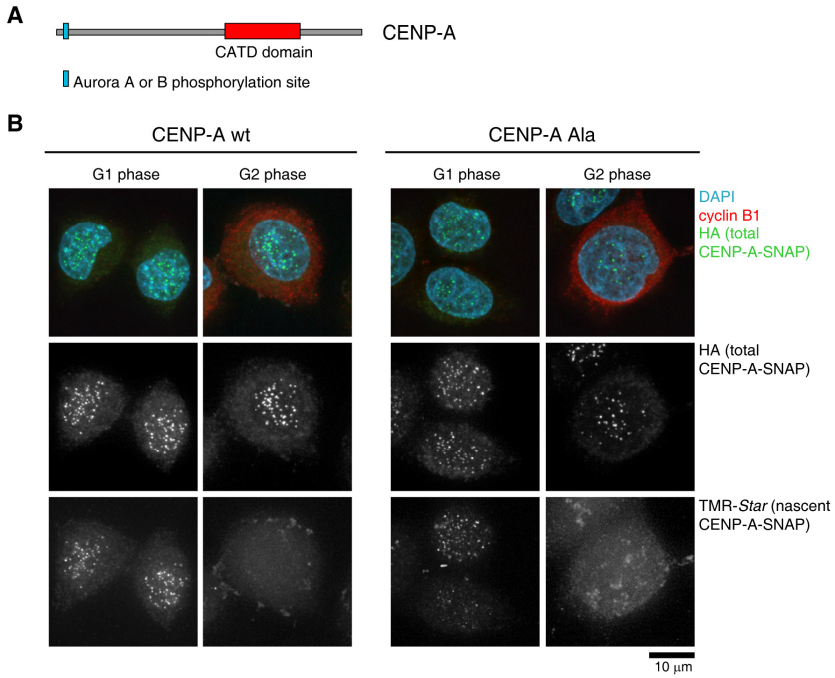

Figure 4.6. Aurora A or Aurora B phosphorylation of Ser 7 of CENP-A is not required for assembly of centromeric chromatin. (A) Schematic of CENP-A protein, with relevant domains and phosphorylation site indicated. (B) Randomly cycling HeLa cells were transfected with SNAP fusions of CENP-A wt and CENP-A carrying a Serine 7 to Alanine mutation. 48 hours after transfection the available CENP-A-SNAP pool was quenched with BTP and 7 hours later the nascent pool was pulse labeled with TMR-*Star*. Representative images of cells in G1 and G2 phase are shown. Cells were fixed and counterstained for cyclin B and with DAPI to indicate G2 status and DNA respectively.

Expression of HJURP lacking a domain that is conserved among vertebrates induces CENP-A assembly in G2 phase

We have shown that G2 phase-induced CENP-A assembly requires the members of the Mis18 complex and HJURP (Figure 4.4 A and B). Additionally, our results suggest that CENP-A loading into centromeric chromatin during G2 phase depends on the centromere targeting of these CENP-A assembly factors, which rapidly occurs upon Cdk inhibition (Figure 4.2 and 4.3). Therefore, Mis18α, Mis18β, Mis18BP1^{HsKNL2} and/or HJURP may contain regulatory domains that control their centromere localization and the timing of CENP-A assembly. We hypothesized that mutants that have lost their potential for cell cycle regulation, but retain the ability to induce CENP-A assembly, may do so even outside G1 phase. To test this, we created a series of deletion mutants of three of these proteins fused to GFP, which are listed in Table A2 in Appendix 1. We screened mutants by transient expression in cells that stably express CENP-A-SNAP, and determined their localization and their ability to induce CENP-A assembly prior to mitosis. None of the deletion mutants of Mis18α was able to localize to centromeres at any stage of the cell cycle, indicating that the domains responsible for its centromere localization are not clustered in any one of the protein fragments. Moreover, none of these mutants induced premature CENP-A assembly (Table A2, Appendix 1). Similarly, most of the deletion mutants of Mis18β tested did not show clear centromeric localization and did not affect the timing of CENP-A assembly (Table A2, Appendix 1). The Mis18β mutant lacking the C-terminal 44 amino acids, localized normally to centromeres from late anaphase to mid G1 phase, but did not change the timing of CENP-A assembly (Table A2, Appendix 1). In the case of HJURP, we found that two N-terminal deletion mutants of HJURP both encompassing the Scm3 homology domain were unable to localize to centromeres (Table A2, Appendix 1). This domain is part of the CENP-A binding domain of HJURP, which consist of its first 80 amino acids (Shuaib et al., 2010). These two mutants are likely no longer part of

CENP-A/H4 prenucleosomal complexes. Therefore, this result suggests that centromere targeting of HJURP may depend on its association with soluble CENP-A. Two additional mutants of HJURP with internal deletions that include the repeated regions of this protein (Figure 4.7 A) were also unable to localize at centromeres (Table A2, Appendix 1), indicating that HJURP has more than one domain responsible for its centromere targeting. Another HJURP deletion mutant, lacking its C-terminal domain, localized to centromeres during late telophase/early G1 (Table A2, Appendix 1), indicating this domain is not required for HJURP targeting to centromeres. Surprisingly, an internal deletion that includes part of the vertebrate conserved domain of HJURP (HJURP$^{\Delta 184-282}$-GFP) (Sanchez-Pulido et al., 2009) caused unscheduled assembly of CENP-A-SNAP at centromeres (Figure 4.7) similar to that observed after Roscovitine treatment. However, the premature CENP-A assembly induced by this mutant was less efficient than Roscovitine-induced one, as only ~10% of transfected cyclin B positive cells showed CENP-A assembly, and only when CENP-A-SNAP was overexpressed. Although HJURP$^{\Delta 184-282}$-GFP localizes properly at centromeres in the majority of late telophase/early G1 phase cells, it was observed at G2 centromeres only in a small subset of cells that presented unscheduled CENP-A assembly (Figure 4.7 C). Indeed, this is expected because HJURP acts to deliver CENP-A at centromeres and it is normally localized at centromeres only during late telophase/early G1 phase, while CENP-A remains incorporated into centromeric nucleosomes throughout the cell cycle (Dunleavy et al., 2009; Foltz et al., 2009). However, part of the reason why the targeting of HJURP$^{\Delta 184-282}$-GFP is less efficient in G2 phase than in G1 phase is likely due to the fact that centromere localization of HJURP depends on the centromeric localization of the Mis18 complex (Barnhart et al., 2011). Since this complex acts upstream of HJURP, it is likely that the transfection of HJURP$^{\Delta 184-282}$-GFP does not recruit the Mis18 complex to centromeres. This suggests that this deletion mutant of HJURP can partially escape the requirement of the priming step executed by the Mis18 complex. However, we expect that under these conditions the CENP-A assembly

pathway is only partially activated, which explains the low efficiency of CENP-A assembly upon HJURP$^{\Delta 184\text{-}282}$-GFP expression. Additionally, we showed that a smaller internal deletion, which comprises the first 54 amino acids of vertebrate conserved domain of HJURP (HJURP$^{\Delta 228\text{-}282}$-GFP), is also able to induce CENP-A assembly during G2 phase (Figure 4.7). This HJURP$^{\Delta 228\text{-}282}$-GFP mutant presents a localization pattern identical to HJURP$^{\Delta 184\text{-}282}$-GFP (Figure 4.7).

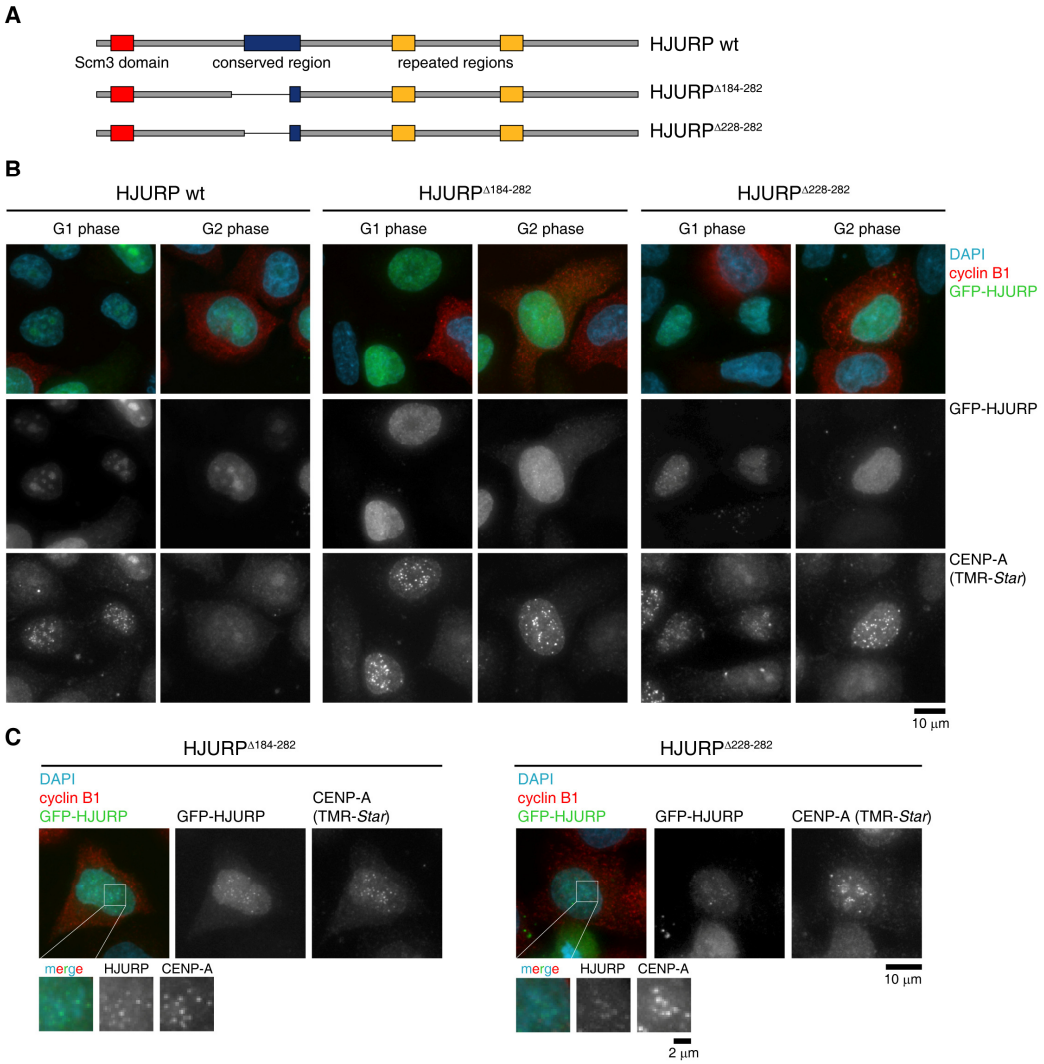

Figure 4.7. Expression of HJURP mutants that lack part of the vertebrate conserved domain induces CENP-A assembly prior to mitosis. Continued on the next page.

Figure 4.7. (continued) (A) Schematic of HJURP wt, HJURP$^{\Delta 184-282}$ and HJURP$^{\Delta 228-282}$ proteins. Relevant domains are indicated. (B) Randomly cycling HeLa cells stably expressing CENP-A-SNAP were transfected with GFP-tagged HJURP constructs (listed in Table A2, Appendix 1). 24 hours after transfection the existent CENP-A-SNAP pool was quenched with BTP and 7 hours later a nascent pool was pulse labeled with TMR-*Star*. Cells were fixed and counterstained for cyclin B and with DAPI to indicate G2 status and DNA respectively. ~100 cells were analyzed and representative images of cells in G1 (low cyclin B) and G2 phase (high cyclin B) are shown. (C) Images of G2 cells in which HJURP$^{\Delta 184-282}$ and HJURP$^{\Delta 228-282}$ was localized at centromeres.

Phosphorylation of Mis18BP1^{HsKNL2} controls its centromere localization

The rapid recruitment of the Mis18 complex and HJURP after Roscovitine treatment suggests that Cdk activity acts either directly or indirectly on these components. It is plausible that one or more of these CENP-A assembly factors are inactivated and maintained in a noncentromeric state through a direct phosphorylation by Cdk or by a downstream kinase. Upon mitotic exit, Cdk is inactivated and the counteracting phosphatases become active. Consequently, these proteins whould lose their phosphorylation, resulting in their activation and targeting to the centromere. Consistently, previous phosphoproteome screens have found that Mis18α, Mis18β, Mis18BP1^{HsKNL2} and HJURP are phosphorylated in one or more residues listed in Table 4.2. Importantly, some of the HJURP and Mis18BP1^{HsKNL2} phosphorylation sites are Cdk consensus sites, indicating that Cdk activity may in fact directly control the centromere localization of these proteins.

To determine which CENP-A assembly factor or factors are regulated through phosphorylation we mutated all (or almost all) known Serine/Threonine phosphorylation sites of Mis18α, Mis18β, Mis18BP1^{HsKNL2} and HJURP to Alanine (Table 4.2).

Table 4.2. Known phosporylation sites on CENP-A assembly factors			
Protein	**Position**	**Cdk consensus**	**References**
Mis18α	S233	No	(Dephoure et al., 2008)
Mis18β	S47	No	(Dephoure et al., 2008)
	S48	Yes	(Dephoure et al., 2008)
	T221	Yes	(Mayya et al., 2009)
	S225	No	(Mayya et al., 2009)
Mis18BP1^{HsKNL2}	S110	Yes	(Dephoure et al., 2008)
	S135	No	(Wang et al., 2008)
	S192	No	(Dephoure et al., 2008)
	T261	No	(Olsen et al., 2006)
	S263	No	(Olsen et al., 2006)
	S299	No	(Wang et al., 2008)
	S365	Yes	(Wang et al., 2008)
	S541	Yes	(Olsen et al., 2006)
	T653	Yes	(Dephoure et al., 2008)
	T821	No	(Dephoure et al., 2008)
	S824	No	(Dephoure et al., 2008)
	S914	Yes	(Dephoure et al., 2008)
	S991	Yes	(Dephoure et al., 2008)
	T993	Yes	(Dephoure et al., 2008)
	S1004	No	(Dephoure et al., 2008)
	S1008	No	(Dephoure et al., 2008)
	S1086	Yes	(Dephoure et al., 2008)
	S1087	Yes	(Dephoure et al., 2008)
	S1104	No	(Dephoure et al., 2008)
HJURP	T122	No	(Dephoure et al., 2008)
	S123	No	(Dephoure et al., 2008)
	S128	No	(Dephoure et al., 2008)
	S140	Yes	(Dephoure et al., 2008)
	S185	Yes	(Cantin et al., 2008)
	S412	Yes	(Dephoure et al., 2008)
	S448	Yes	(Dephoure et al., 2008)
	S473	Yes	(Dephoure et al., 2008)
	S486	No	(Luhn et al., 2007)
	S642	Yes	(Dephoure et al., 2008)

Mutation of the unique phosphorylation site in Mis18α did not alter the timing of its centromere localization nor the timing of CENP-A assembly (Figure 4.8). We observed that Mis18α^{S233A} and nascent CENP-A-SNAP were localized at centromeres in G1 phase, but not in G2 phase (Figure 4.8).

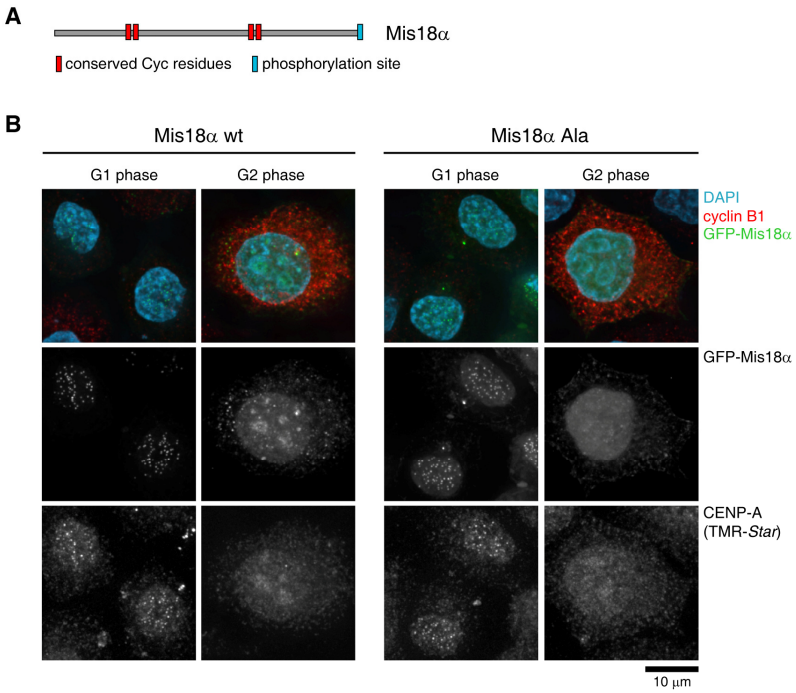

Figure 4.8. Phosphorylation of Mis18α at Serine 233 is not required for its centromere localization. (A) Schematic of Mis18α protein, with relevant domains and known phosphorylation site indicated. (B) Randomly cycling HeLa cells stably expressing CENP-A-SNAP were transfected with GFP fusions of Mis18α wt and Mis18α^{S233A} proteins (Table 4.2). 24 hours after transfection the existing CENP-A-SNAP pool was quenched with BTP and 7 hours later a nascent pool was pulse labeled with TMR-*Star*. Cells were fixed and counterstained for cyclin B and with DAPI to indicate G2 status and DNA respectively. ~100 cells were analyzed and representative images of cells in G1 and G2 phase are shown.

Similarly, mutation of all phosphorylation sites in Mis18β to Alanine had no effect on its centromere localization in G1 phase and did not alter the timing of CENP-A assembly (Figure 4.9). However, we observed that expression of either Mis18β wt or Mis18β Alanine mutant caused a reduced efficiency of CENP-A assembly when compared to expression of other CENP-A assembly factors such as Mis18α (Figure 4.8 and 4.9). This result suggests that overexpression of Mis18β may have a negative effect on CENP-A incorporation into centromeric chromatin.

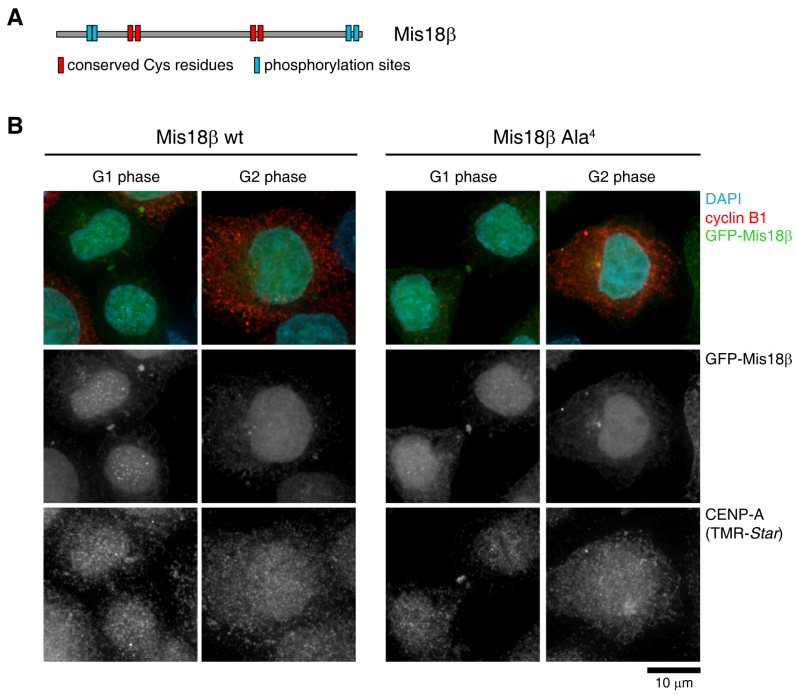

Figure 4.9. Phosphorylation of Mis18β is not required for its centromere localization. (A) Schematic of Mis18β protein, with relevant domains and known phosphorylation sites indicated. (B) Randomly cycling HeLa cells stably expressing CENP-A-SNAP were transfected with GFP fusions of Mis18β wt and Mis18β-Ala^4 mutant (Table 4.2). 24 hours after transfection the existing CENP-A-SNAP pool was quenched with BTP and 7 hours later a nascent pool was pulse labeled with TMR-*Star*. Cells were fixed and counterstained for cyclin B and with DAPI to indicate G2 status and DNA respectively. ~100 cells were analyzed and representative images of cells in G1 and G2 phase are shown.

In contrast, to Mis18α or β, mutation of 8 of the 10 known phosphorylation sites of HJURP impaired its centromere localization (HJURP-Ala^8, a mutant with all known phosphorylation sites modified to Alanine, except S185 and S486). Transfections of HJURP-Ala^8 mutant did not affect CENP-A loading in G1 phase (Figure 4.10), suggesting that, in addition to its failure to localize, this mutant also did not exert a dominant negative effect on canonical CENP-A assembly.

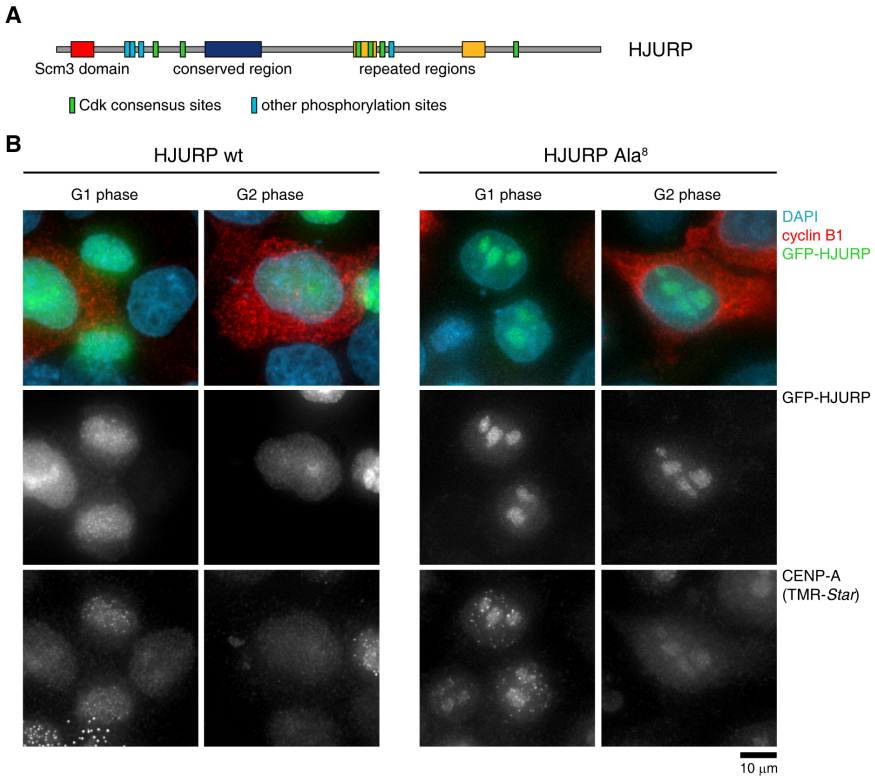

Figure 4.10. Phosphorylation of HJURP is required for its centromere localization. (A) Schematic of HJURP protein with relevant domains and known phosphorylation sites indicated. (B) Randomly cycling HeLa cells stably expressing CENP-A-SNAP were transfected with GFP fusions of HJURP wt and HJURP-Ala^8 [a mutant with all known phosphorylation sites modified to Alanine, except S185 and S486 (Table 4.2)]. 24 hours after transfection the existing CENP-A-SNAP pool was quenched with BTP and 7 hours later a nascent pool was pulse labeled with TMR-*Star*. Cells were fixed and counterstained for cyclin B and with DAPI to indicate G2 status and DNA respectively. ~100 cells were analyzed and representative images of cells in G1 and G2 phase are shown.

Strikingly however, conversion of 24 Serine and Threonine residues in Mis18BP1^{HsKNL2} to Alanine (20 known phosphorylation sites plus an additional 4 Serine/Threonine residues immediately adjacent these known phosphorylation sites; Mis18BP1^{HsKNL2}-Ala^{24}; Figure 4.11 A) did not interfere with G1 phase localization to centromeres and resulted in a precocious recruitment of this protein to centromeres in G2 phase and mitosis (Figure 4.11 B and 4.11 C). Nearly half of these phosphorylation sites are Cdk consensus sites (Figure 4.11 A), indicating that Cdk activity may indeed directly control the centromere localization of Mis18BP1^{HsKNL2}. Consistent with this hypothesis, we observed that Mis18BP1^{HsKNL2} is phosphorylated in a cell cycle dependent manner, most prominently when cells are enriched in mitosis, the cell cycle phase during which Cdk activity is highest (Figure 4.11 D, left). Dephosphorylation of Mis18BP1^{HsKNL2} by phosphatase treatment *in vitro* or by mutation of known sites (Mis18BP1^{HsKNL2}-Ala^{24}) resulted in the loss of high molecular weight species, indicating that most, if not all, phosphorylation events are removed in the 24 Alanine mutant we generated (Figure 4.11 D, right). These results strongly suggest that Mis18BP1^{HsKNL2} is kept in an inactive, noncentromeric state by phosphorylation. This inhibitory state is alleviated by loss of phosphorylation during mitotic exit, when Cdk activity is lost. Although mutation of 24 Serine/Threonine residues in Mis18BP1^{HsKNL2} to Alanine resulted in its premature targeting to centromeres, it did not induce unscheduled CENP-A assembly (Figure 4.12). These results indicate that Cdk mediated inhibition of CENP-A assembly is not exclusively exerted by controlling centromere localization of Mis18BP1^{HsKNL2}. Instead, it is likely that Cdk activity inhibits assembly of centromeric chromatin by controlling the centromere targeting of more than one key CENP-A assembly factor.

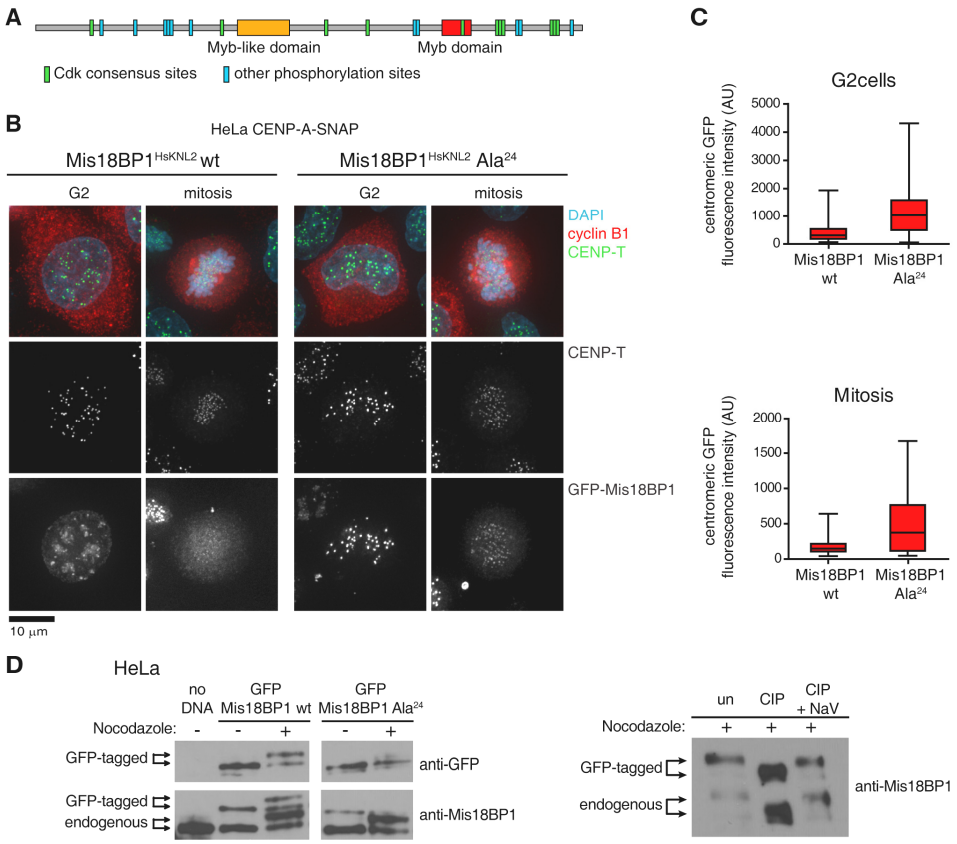

Figure 4.11. Phosphorylation of Mis18BP1^{HsKNL2} controls its centromere localization. (A) Schematic of Mis18BP1^{HsKNL2} protein with relevant domains and known phosphorylation sites indicated. (B) Constructs expressing wild type GFP-tagged Mis18BP1^{HsKNL2} or Mis18BP1^{HsKNL2}-Ala^{24} were transfected into asynchronous HeLa cells 32 hours prior to fixation followed by counterstaining for cyclin B, CENP-T and with DAPI to indicate G2 status, centromeres and DNA, respectively. (C) Box and whisker plots of relative GFP-Mis18BP1^{HsKNL2} fluorescent signal per centromere in G2 phase (high cyclin B) and mitotic cells. CENP-T was used as a reference for centromere position. >300 and >90 centromeres were quantified in G2 and mitotic cells, respectively. (D) Left: HeLa cells transiently expressing GFP-tagged Mis18BP1^{HsKNL2} or Mis18BP1^{HsKNL2}-Ala^{24} for 29 hours were left untreated or were treated with Nocodazole for another 12 hours to enrich for mitotic cells, followed by processing for SDS-PAGE and immunoblotting. Endogenous and GFP-tagged Mis18BP1^{HsKNL2} is detected by indicated antibodies. High molecular weight species are detected in Nocodazole treated cells. Continued on the next page.

Figure 4.11. (continued) (D) Right: GFP-tagged Mis18BP1^{HsKNL2} was pulled down from mitotic cell extracts that were either untreated (un), treated with Calf Intestinal Alkaline Phosphatase (CIP) alone or in combination with Sodium Orthovanadate (NaV), followed by SDS-PAGE and immunoblotting for Mis18BP1^{HsKNL2}.

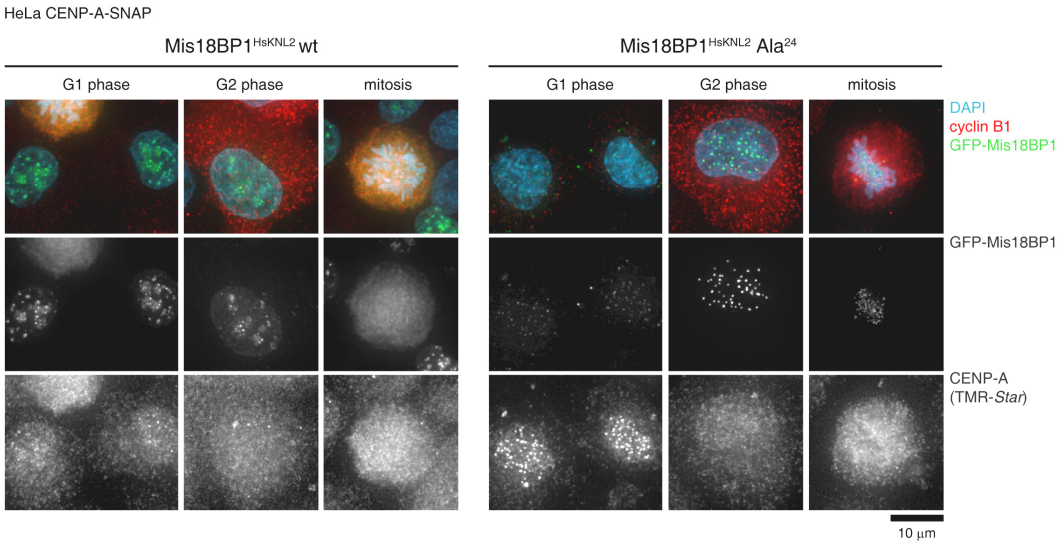

Figure 4.12. Preventing phosphorylation of Mis18BP1^{HsKNL2} results in its targeting to centromeres in G2 phase and mitosis but is not sufficient to induce CENP-A assembly. Constructs expressing wild type GFP-tagged Mis18BP1^{HsKNL2} or Mis18BP1^{HsKNL2}-Ala^{24} were transfected into asynchronously cycling HeLa cells stably expressing CENP-A-SNAP 32 hours prior to fixation. 24 hours after transfection cells were subjected to quench-chase-pulse labeling as described in Chapter 2. After the labeling step with TMR-*Star* cells were fixed and counterstaining for cyclin B, CENP-T and with DAPI to indicate G2 status, centromeres and DNA, respectively.

Discussion and Conclusions

CENP-A assembly into centromeric chromatin is uncoupled from DNA replication and is restricted to late mitosis/early G1 phase of the cell cycle (Shelby et al., 2000; Jansen et al., 2007; Schuh et al., 2007; Hemmerich et al., 2008; Bernad et al., 2011; Moree et al., 2011). Centromere targeting of the CENP-A assembly machinery, which includes the Mis18 complex and HJURP, is also temporal controlled and restricted to anaphase and late telophase, respectively (Chapter 2). Initially, we have demonstrated that the combined activity of Cdk1 and Cdk2 controls the timing of CENP-A assembly at centromeres (Chapter 3). Here, we determined that CENP-A assembly in G2 phase, induced by Cdk inhibition, requires centromere targeting of the Mis18 complex and HJURP (Figure 4.2 – 4.4). We also provide evidence that Aurora A and Aurora B, two mitotic kinases downstream of Cdk activity, are neither required for CENP-A assembly nor for its cell cycle regulation (Figure 4.5 and 4.6). Additionally, we showed that a domain of HJURP conserved among vertebrates contributes to cell cycle control of CENP-A assembly. Expression of a mutant lacking this domain resulted in premature deposition of CENP-A into centromeric chromatin (Figure 4.7). Finally, we established that phosphorylation of Mis18BP1^{HsKNL2} controls its centromere localization (Figure 4.11). Thus, Cdk activity may regulate the timing of CENP-A assembly through phosphorylation of Mis18BP1^{HsKNL2} and perhaps of other components of the CENP-A assembly machinery.

The HJURP vertebrate conserved domain is an important regulatory domain required for cell cycle control of CENP-A assembly

We showed that force expression of HJURP lacking part of its vertebrate conserved domain (HJURP$^{\Delta 184-282}$-GFP or HJURP$^{\Delta 228-282}$-GFP) caused unscheduled CENP-A assembly (Figure 4.7). We observed that CENP-A-SNAP loading and HJURP targeting to centromeres is less robust then following inhibition of Cdk activity, suggesting that HJURP$^{\Delta 184-282}$-GFP and HJURP$^{\Delta 228-282}$-GFP create conditions that allow for only partial activation of CENP-A assembly, while cell cycle control of assembly has been impaired. This low efficiency can be in part explained by the fact that HJURP targeting to centromeres requires licensing of centromeric chromatin by the Mis18 complex (Barnhart et al., 2011). Remarkably, centromere targeting of HJURP$^{\Delta 184-282}$-GFP and HJURP$^{\Delta 228-282}$-GFP apparently escapes the need for Mis18-mediated licensing of CENP-A assembly, albeit only partially. This also explains why these mutants are properly localized to the centromeres during late anaphase when Mis18 is present at the centromere. Although we have not shown that the Mis18 complex is not recruited under these conditions in G2 phase, we speculate that CENP-A assembly can occur in the absence of the Mis18 complex, which until now was thought to be essential for this process.

Taken together, these results lead us to hypothesize that the loss of cell cycle regulation of centromeric localization of HJURP$^{\Delta 184-282}$-GFP or HJURP$^{\Delta 228-282}$-GFP is caused by their inability to be phosphorylated by Cdk or by a downstream kinase. Indeed, the HJURP$^{\Delta 184-282}$-GFP mutant lacks Serine 185 that is known to be phosphorylated *in vivo* (Table 4.2). However, this residue is preserved in the deletion mutant HJURP$^{\Delta 228-282}$-GFP which also induces CENP-A assembly in G2 phase and contains all known phosphorylation sites (Figure 4.7 and Table 4.2). Yet, it is possible that the deleted region serves as a docking site for a kinase or that conformational changes caused by this deletion impair the interaction with Cdk or other kinases responsible for its phosphorylation.

Consistent with this, HJURP$^{\Delta 184-282}$-GFP and HJURP$^{\Delta 228-282}$-GFP lack a RXL motif, which is known to increase the specificity of recognition of Cdk substrates through direct binding by cyclin A and cyclin E. HJURP has in total five RXL motifs that may be required for its Cdk phosphorylation. It is possible that loss of one of these motifs will decrease the efficiency of Cdk phosphorylation, which may lead to partial loss of cell cycle control of its centromere localization and activity.

An alternative explanation of our observations is that HJURP$^{\Delta 184-282}$-GFP is no longer able to promote CENP-A assembly but acts as a dominant negative sink for inhibitory phosphorylation, inducing the unscheduled activation and centromere recruitment of endogenous HJURP and consequent CENP-A assembly. This model would be consisted with the preservation of all known phosphorylation sites in our deletions mutants. Determining whether HJURP$^{\Delta 184-282}$-GFP is still phosphorylatable *in vivo* and is able to support CENP-A assembly in the absence of endogenous HJURP is expected to differentiate between these alternative models.

A recent study in *Xenopus* identified two closely related isoforms of Mis18BP1 (xMis18BP1-1 and xMis18BP1-2) (Moree et al., 2011). The two isoforms have different patterns of localization: xMis18BP1-1 is localized at centromeres during metaphase and G1 phase while xMis18BP1-2 is only centromere localized during G1 phase. Interestingly, the isoform that is localized at centromeres prior to mitosis lacks a domain of close to 50 amino acids, when compared with the one that is only localized during G1 phase (Moree et al., 2011). These observations resemble our results with HJURP lacking the conserved vertebrate domain. In both cases, natural or synthetic deletion of a small domain in Mis18BP1 or HJURP, respectively, cause targeting of these proteins to centromeres prior to G1 entry. This suggests, that the missing regions are regulatory domains that control the timing of centromere targeting of these two CENP-A assembly factors.

CENP-A assembly is activated upon mitotic exit by Cdk inactivation and dephosphorylation of Mis18BP1^{HsKNL2}

Premature assembly of CENP-A in G2 phase is induced by Cdk inhibition and requires centromere recruitment of Mis18α, Mis18β, Mis18BP1^{HsKNL2} and HJURP (Figure 4.2 – 4.4). Here we propose that Cdk activity inhibits the centromere localization of CENP-A assembly factors either through direct phosphorylation or through a cascade with one or more of these proteins as downstream effectors. In agreement with this model, a mutant of Mis18BP1^{HsKNL2} that can no longer be phosphorylated is recruited to centromeres during G2 phase and mitosis (Figure 4.11). Although prevention of phosphorylation of Mis18BP1^{HsKNL2} through Alanine mutations caused its premature targeting to centromeres, it was not able to induce CENP-A assembly prior to G1 phase (Figure 4.12). This observation suggests that Cdk-mediated inhibition of CENP-A assembly may involve the phosphorylation and consequent inhibition of not only Mis18BP1^{HsKNL2} but also other CENP-A assembly factor(s). Alternatively, control of CENP-A assembly timing is mediated solely through Mis18BP1^{HsKNL2} but the residues mutated in Mis18BP1^{HsKNL2}-Ala^{24} may be necessary for its role in the centromere licensing step of CENP-A assembly. To discriminate between these possibilities we are currently testing whether the Mis18BP1^{HsKNL2}-Ala^{24} mutant is able to support canonical CENP-A assembly in the absence of wild type Mis18BP1^{HsKNL2}. Nevertheless, our observation that timing of CENP-A assembly can be affected by expression of two HJURP deletion mutants, argues in favor of the notion that multiple targets of Cdk control exists. We therefore favor a model in which Cdk1 and Cdk2 phosphorylate Mis18BP1^{HsKNL2} and other factor(s), which may include HJURP and/or additional unknown proteins (represented as factor X and Y in Figure 4.13). These phosphorylated proteins are maintained in an inactive, noncentromeric state. During mitotic exit, Cdk1 and Cdk2 are inhibited and these proteins are no longer phosphorylated, which allows their centromere recruitment and CENP-A assembly (Figure 4.13). In Chapter 3 we

demonstrated that CENP-A assembly induced in G2 phase does not require protein synthesis nor protein degradation. Therefore, it is likely that dephosphorylation of Mis18BP1^{HsKNL2} and other CENP-A assembly factors, and consequent activation of CENP-A assembly, requires the activity of a yet to be identified phosphatase.

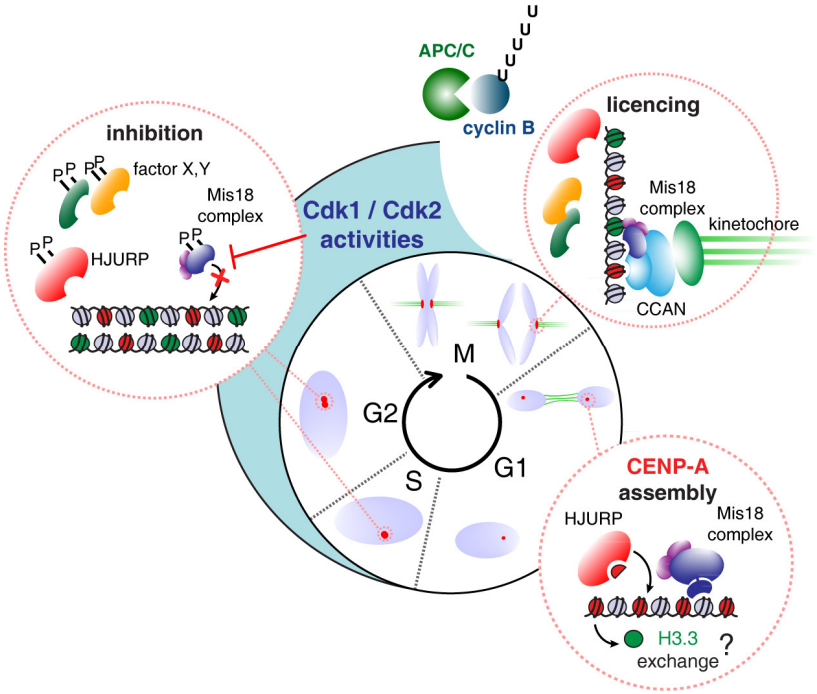

Figure 4.13. Cdk1 and Cdk2 activities maintain the CENP-A assembly machinery in an inactive non-centromeric state through phosphorylation of Mis18BP1^{HsKNL2} and possibly other components. Cartoon illustrates Cdk1/Cdk2-mediated inhibition of CENP-A assembly, accomplished in part through phosphorylation (P) of Mis18BP1^{HsKNL2} (member of the Mis18 complex) during S, G2 and M phases. Phosphorylation of HJURP may also be required for this inhibition. Factors X and Y symbolize the potential involvement of other, yet to be identified, components. Inhibition is alleviated trough APC/C mediated loss of Cdk1 activity in anaphase, targeting the Mis18 complex to the centromere (licensing) followed by HJURP targeting and CENP-A assembly in G1 phase. CENP-A assembly in G1 phase possibly involves an exchange with H3.3 (Dunleavy et al., 2011). Canonical (H3.1 containing) nucleosomes are shown in light blue, H3.3 nucleosomes in green and CENP-A nucleosomes in red.

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Chapter 5 – General Discussion

Epigenetic inheritance of centromeres

The demonstration that CENP-A is required and sufficient to nucleate a heritable and functional centromere in human and fly cells places it at the heart of the epigenetic mechanism responsible for propagation and maintenance of centromere identity (Barnhart et al., 2011; Mendiburo et al., 2011; Olszak et al., 2011). Thus, determining how this epigenetic mark is replicated and maintained becomes crucial to understand how centromeres are epigenetically inherited across cell divisions. During the last decade, there has been a significant advance in answering this question. Many proteins have been identified to play a crucial role in assembling CENP-A into centromeric chromatin, including the Mis18 complex (Fujita et al., 2007; Maddox et al., 2007) and the CENP-A chaperone HJURP (Dunleavy et al., 2009; Foltz et al., 2009; Shuaib et al., 2010) It has also been demonstrated that CENP-A chromatin is able to propagate itself (Mendiburo et al., 2011), and that this self-propagation mechanism is cell cycle regulated, restricting CENP-A assembly to late mitosis/early G1 phase in metazoans (Jansen et al., 2007; Schuh et al., 2007; Hemmerich et al., 2008; Bernad et al., 2011; Moree et al., 2011). These observations raised an important question: How is centromere propagation cell cycle controlled? The work presented in this thesis provides a significant advance on answering this question and identifies new players in the CENP-A assembly pathway (Carroll et al., 2009; Silva et al., 2012; Stimpson and Sullivan, 2012).

In chapter 2, we show that structural components of the centromere, which include CENP-C, CENP-T and CENP-N, are required for CENP-A assembly, but their specific roles in this process remain unclear (Figures 2.4 and 2.5). One likely role for these proteins may be to form a recognition platform required for targeting the Mis18 complex to centromeres (Moree et al., 2011; Dambacher et al., 2012). In chapters 3 and 4 we shed light on the cell cycle control mechanism responsible for centromere propagation. In this chapter, I will discuss how centromere propagation is cell cycle regulated by Cdk activity in humans and in

other organisms. I will also discuss an important question raised by the results presented in this thesis: Why is CENP-A assembly cell cycle controlled? Finally, I will discuss the implications of our findings for general epigenetic inheritance and human disease.

Cdk activity couples epigenetic inheritance of the centromere with cell cycle progression

The timing of CENP-A assembly coincides with the conclusion of mitosis. However, the mitotic signal that initiates centromere propagation has been unclear until now. Many hypotheses have been proposed during the last decade that highlight the possible role of important mitotic events in initiating CENP-A assembly [reviewed in Chapter 1, Section 3.2.2; (Mellone and Allshire, 2003; Jansen et al., 2007; Allshire and Karpen, 2008; Brown and Xu, 2009; Pauleau and Erhardt, 2011)]. The work presented in this thesis reveals that the only mitotic event required for initiating CENP-A assembly is the APC/C-mediated degradation of cyclins A and B and the resulting loss of Cdk activity. Premature loss of Cdk1 and Cdk2 activities is sufficient to induce CENP-A assembly prior to mitosis (Figure 3.13). Unscheduled CENP-A assembly requires centromere localization of the Mis18 complex and HJURP, which are present throughout the cell cycle but are kept in an inactive noncentromeric state by Cdk activity (Figures 4.2 – 4.4). Moreover, loss of Cdk activity triggers CENP-A assembly independently of APC/C-mediated proteolysis and of protein synthesis. These observations suggest that Cdk-mediated phosphorylation of one or more CENP-A assembly factors prevent the targeting of these proteins to the centromere, thereby inhibiting CENP-A assembly. In agreement, we show that Mis18BP1^{HsKNL2} is phosphorylated *in vivo* and that a mutant version of this protein which can no longer be phosphorylated is targeted to the centromere during G2 phase and mitosis (Figure 4.11). However, the unscheduled targeting of Mis18BP1^{HsKNL2} is not sufficient to drive premature CENP-A assembly (Figure

4.12). Therefore, we propose a model in which Cdk1 and Cdk2 phosphorylate Mis18BP1^{HsKNL2} and possibly other CENP-A assembly factors, such as HJURP or a yet to be identified protein, preventing their centromere targeting and activation of the CENP-A assembly pathway. Loss of Cdk1 and Cdk2 activities upon APC/C activation during mitotic exit alleviates this inhibitory phosphorylation and allows the centromere recruitment of the CENP-A assembly machinery and initiation of CENP-A assembly (Figure 4.13).

As discussed in the introduction, both kinase and phosphatase activities have important functions in modulating the phosphorylation status of distinct mitotic substrates (reviewed in Chapter 1, Section 4.3). The observation that assembly of CENP-A, following loss of Cdk activity, does not require *de novo* protein synthesis indicates that phosphatase activity may be involved in modifying the phosphorylation status of CENP-A assembly factors. Indeed, different phosphatases of the PP1 and PP2A families are known to contribute to the reversal of Cdk-mediated phosphorylation events (reviewed in Wurzenberger and Gerlich, 2011). The catalytic subunits of these phosphatases have low substrate specificity and are constitutively active. However, the association of these catalytic subunits with a wide range of regulatory subunits modulates the substrate specificity, the intracellular localization and the overall activity of PP1 and PP2A phosphatases (Virshup and Shenolikar, 2009; Wurzenberger and Gerlich, 2011). Since these phosphatases can be constitutively active, it is possible that phosphate groups on one or more CENP-A assembly factors continuously turn over throughout the cell cycle and that the loss of Cdk activity during mitotic exit tips the balance towards dephosphorylation. Once dephosphorylated these CENP-A assembly factors are targeted to centromeres and activate the entire CENP-A assembly pathway.

Alternatively, it is possible that Cdk inhibition promotes phosphatase activity and consequent dephosphorylation of one or more members of the CENP-A assembly machinery. Interestingly, it has been proposed that PP2A-B55α activity can be inhibited through Cdk1-mediated phosphorylation (Wurzenberger and

Gerlich, 2011). B55α is a regulatory subunit of PP2A and is phosphorylated *in vivo* at Serine 167, which is part of a Cdk substrate motif (S-P-X-R). A phospho-mimicking mutant of B55α, in which Serine 167 was replaced by Glutamate (Ser167Glu), binds less efficiently to PP2A (Schmitz et al., 2010), suggesting that formation of a functional PP2A-B55α complex may be controlled by a Cdk-dependent phosphorylation of B55α. It is conceivable that PP2A activity, triggered by inhibition of Cdk activity, has an active role in the CENP-A assembly pathway by dephosphorylating proteins required to incorporate CENP-A into centromeric chromatin.

Importantly, Cdk1-cyclin B activity also inhibits PP1γ activity through phosphorylation of its regulatory subunit Repo-Man. This phosphorylation of Repo-Man prevents its association with PP1γ, similar to what was described for PP2A-B55α (Vagnarelli et al., 2011). Dephosphorylation of Repo-Man during anaphase allows its association with PP1γ, and targets this active phosphatase complex to mitotic chromatin (Trinkle-Mulcahy et al., 2006; Vagnarelli et al., 2006, 2011). These observations point to PP1γ-Repo-Man as another good candidate for dephosphorylation of one or more CENP-A assembly factors at chromatin during mitotic exit. Thus, besides its role in chromosome organization and nuclear envelope reassembly (Vagnarelli et al., 2011), PP1γ-Repo-Man may also have an important role in centromere propagation. Specific inhibition of PP2A and PP1γ using Thyrsiferyl 23-acetate or Tautomycetin, respectively (Matsuzawa et al., 1994; Mitsuhashi et al., 2003) should determine if and which of these phosphatases are involved in triggering CENP-A assembly.

In summary, combination of the kinase activities of Cdk1 and Cdk2 with the phosphatase activity of PP1γ and/or PP2A, or other unknown phosphatase, may play a crucial role in temporally controlling the phosphorylation status of Mis18BP1^{HsKNL2} and of other proteins involved in centromere propagation, for example HJURP.

Cdk activity controls duplication of DNA, centrosomes and centromeres

Our work revealed that centromere propagation is regulated by the general cell cycle control machinery that also regulates other cellular duplication processes such as DNA replication and centrosome duplication. There are broad parallels between the way these different processes are cell cycle regulated. The oscillations of Cdk activity across the cell cycle allow these important processes to occur properly, sequentially and in synchrony with cell division. All these duplication events occur in two steps that are differentially regulated by Cdk activity. In the case of DNA replication, low Cdk activity upon mitotic exit promotes licensing of the replication origins by triggering the assembly of the pre-replicative complexes (pre-RCs). In contrast, high Cdk2-cyclin E activity during S phase allows the firing of those origins and prevents pre-RC reassembly. In the case of centrosome duplication, mitotic exit and downregulation of Cdk activity are required for centriole disengagement (Tsou and Stearns, 2006), which licenses centriole duplication during the next S phase. The high Cdk2-cyclin E activity during this phase of the cell cycle promotes duplication and growth of centrioles, and prevents their disengagement. In this thesis, we demonstrated that loss of Cdk activity during anaphase is required for CENP-A assembly. On the other hand, high Cdk activity allows replication of centromeric chromatin and thereby creation of CENP-A nucleosome binding sites, but blocks CENP-A assembly. In summary, while high Cdk activity promotes centriole duplication, initiation of DNA replication and replication of centromeric chromatin, low Cdk activity allows licensing of DNA replication, centriole disengagement and centromere propagation. Thus, Cdk activity is responsible for the temporal separation of two crucial steps of DNA, centrosome and centromere duplication, and renders them mutually exclusive. This ensures that these processes are initiated only once per cell cycle. Importantly, Cdk activity also marks the temporal window of opportunity during which duplication of DNA, centrosomes

and centromeres occurs, thereby preventing over duplication of these cellular components.

Although these duplication processes are all regulated by Cdk activity, the molecular mechanisms downstream of Cdks can vary for each process. In the case of centrosome duplication, high Cdk activity prevents centriole disengagement by inhibiting the APC/C and thus preventing the activation of the protease separase (through degradation of its inhibitor, Securin) (Tsou and Stearns, 2006; Steere et al., 2011). Once securin is degraded by APC/C-mediated proteolysis the centrioles disengage and are licensed for duplication, which requires high Cdk2-cyclin E activity (Hinchcliffe et al., 1999; Matsumoto et al., 1999).

In the case of DNA replication, high Cdk activity inhibits licensing of replication by preventing the recruitment of pre-RC complex, which includes the ORC complex, Cdc6, Cdt1 and the Mcm2-7 complex, to replication origins (Bell and Dutta, 2002; Arias and Walter, 2007). In budding yeast, Cdk^{Cdc28} inhibits pre-RC assembly by direct phosphorylation of each one of the pre-RC components (reviewed in Chapter 1, Section 4.1.4 and Arias and Walter, 2007; Tanaka and Araki, 2010). In vertebrates, in addition to phosphorylation of some pre-RC components, it involves the APC/C-mediated degradation of geminin, an inhibitor of pre-RC formation (reviewed in Chapter 1, Section 4.1.4 and Arias and Walter, 2007; Porter, 2008).

In this thesis we demonstrated that Cdk activity prevents centromere propagation by inhibiting the centromere targeting of important CENP-A assembly factors, such as the Mis18 complex and the CENP-A chaperone HJURP. We have presented evidence that Cdk inhibits centromere propagation, in part, through phosphorylation of $Mis18BP1^{HsKNL2}$ (Figure 4.11). However, preventing phosphorylation of $Mis18BP1^{HsKNL2}$ is not sufficient to initiate centromere propagation (Figure 4.12). This suggests that, similar to the way it inhibits licensing of DNA replication, Cdk may prevent CENP-A assembly through direct phosphorylation of several proteins required for CENP-A assembly.

However, in contrast to DNA replication and centrosome duplication, propagation of centromeric chromatin does not require proteolysis of an inhibitory protein. Instead, it appears to rely exclusively on a Cdk mediated phospho-switch that results in the centromere targeting of proteins that are required for CENP-A deposition into centromeric chromatin.

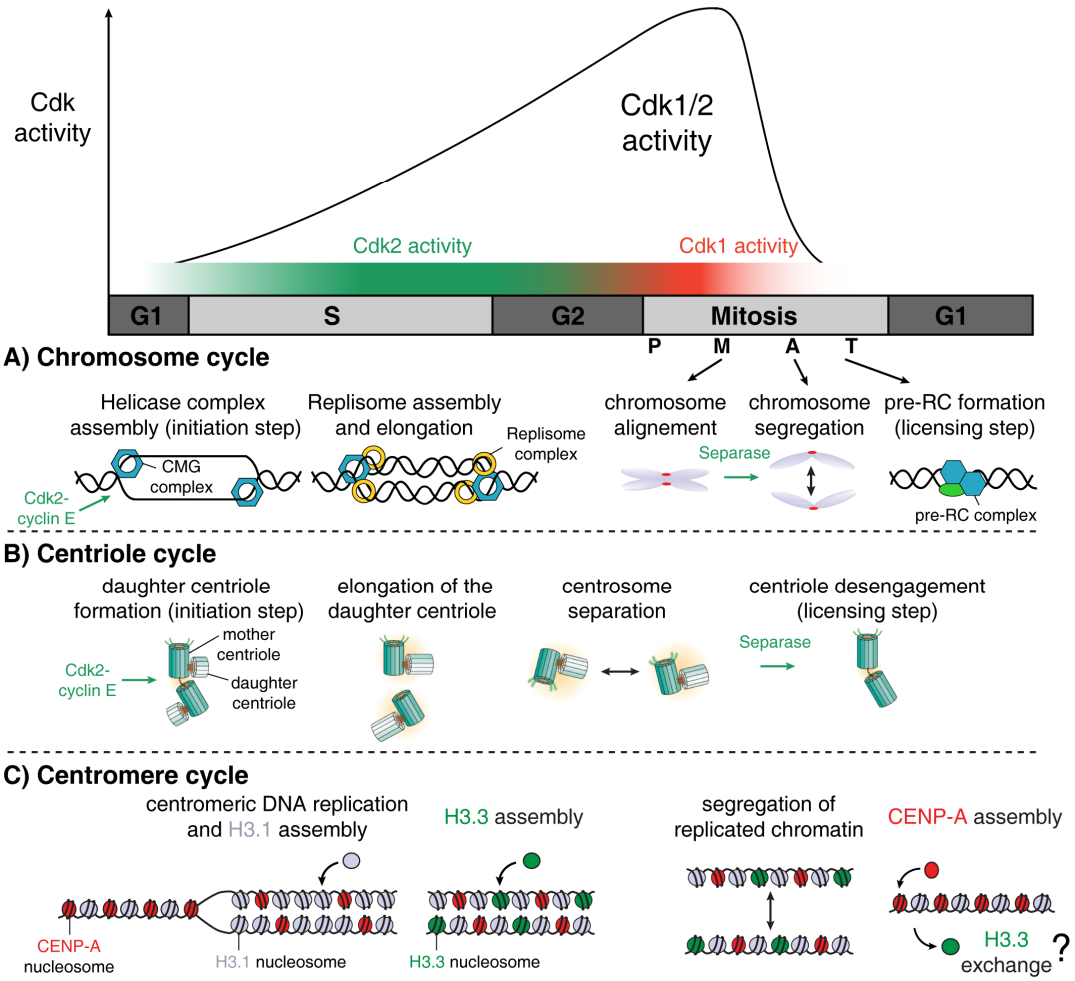

Figure 5.1. Cartoon illustrating how Cdk activity coordinates the duplication of DNA, centrioles and centromeres with cell cycle progression. At the onset of S phase Cdk2-cyclin E activity promotes initiation of DNA replication (A), daughter centriole formation (B), and replication coupled assembly of histone H3.1 (C). Continued on the next page.

Figure 5.1. (continued) Throughout S phase the replisome complex drives synthesis and elongation of the new DNA strands (A), daughter centrioles elongate (B), replication coupled assembly of H3.1 continues and replication uncoupled assembly of histone H3.3 occurs (C). During S, G2 and early M phases, high CDK activity prevents pre-RC assembly, centriole disengagement and CENP-A assembly. During mitotic exit Cdk activity drops and APC/C-mediated degradation of securin leads to separase activation, which allows chromosome segregation and centriole disengagement (A and B). In addition APC/C-mediated loss of geminin leads to reassembly of pre-RC (A). These events license centrioles and DNA for another round of duplication. Loss of Cdk activity at the end of mitosis also promotes CENP-A assembly (C), which continues until the end of G1, when Cdk2 activity starts to rise. CENP-A assembly likely involves exchange with histone H3.3 containing nucleosomes (Dunleavy et al., 2011). The centriole drawings were adapted from Bettencourt-Dias and Glover, 2007.

Intriguingly, Cdk inhibition with the pan-Cdk inhibitor Roscovitine in HeLa cells was not sufficient to induce CENP-A assembly during S phase (Figure 3.8). In chicken DT40 cells, although S phase cells are able to load CENP-A at centromeres, they appear to be less permissive for CENP-A assembly than G2 cells, even after direct inhibition of Cdk1 and Cdk2 (Figures 3.14 – 3.16). This raises the possibility that prevention of CENP-A assembly during S phase may be regulated by an additional mechanism other than Cdk-mediated inhibitory phosphorylation of CENP-A assembly factors. It is possible that during S phase, an inhibitor of CENP-A assembly exists that requires degradation, similar to the requirement for geminin or securing degradation for the licensing of DNA replication and centriole duplication, respectively. Another possibility is that CENP-A assembly factors depend on Cdk1- and/or Cdk2-mediated activating phosphorylation during S phase, which is required for full activation of CENP-A assembly once inhibitory phosphorylation is resolved in G1 phase. The existence of such activating phosphorylation of CENP-A assembly factors would explain why premature inhibition of Cdk activity in S phase would not (fully) activate the CENP-A assembly machinery.

Why is CENP-A assembly cell cycle regulated?

In many eukaryotes, exit from mitosis is a prerequisite for CENP-A assembly (Silva and Jansen, 2009). We have demonstrated that the concomitant loss of Cdk1 and Cdk2 activities during mitotic exit allows centromere targeting of the CENP-A assembly machinery and drives centromere propagation (Chapter 3 and Silva et al., 2012). However, the reason why CENP-A assembly is restricted to this cell cycle window in metazoans remains unknown. One explanation may be to ensure the physical and temporal separation of centromeric chromatin assembly from general chromatin assembly. Limiting expression of CENP-A to S phase results in uniform incorporation of this histone throughout chromosome arms (Shelby et al., 1997), indicating that CENP-A can be assembled by the general chromatin assembly complex CAF1. Limiting CENP-A assembly to a window outside S phase may therefore help restricting CENP-A to the centromere. However, this does not explain why it is delayed until completion of mitosis.

During S phase, while DNA is being replicated, CENP-A nucleosomes are semiconservatively distributed between the two sister chromatids. During S phase histones H3.1 and H3.3 accumulate among parental CENP-A nucleosomes (Dunleavy et al., 2011). Importantly, nucleosomes containing the H3K4me2 mark, which is another conserved feature of centromeric chromatin in humans, flies and yeast, are also maintained in the interspersed domains formed during S phase (Sullivan and Karpen, 2004; Dunleavy et al., 2011). The importance of the mixed chromatin state that contains H3.1, H3.3 and CENP-A nucleosomes is not well understood. Recently, it was shown that the presence of H3 nucleosomes containing the H3K4me2 mark at centromeres is necessary for centromere targeting of HJURP and for CENP-A assembly (Bergmann et al., 2011). Although modified histone H3 assembled in S phase may facilitate subsequent CENP-A assembly, this in itself does not explain the necessity for assembly in G1 phase.

Cells assemble functional kinetochores and progress through mitosis normally with only half of the full complement of CENP-A. This suggests that the mixed CENP-A/H3 chromatin domain may be important for proper kinetochore formation and for chromosome segregation. Accordingly, altering the CENPA:H3 ratio at fission yeast centromeres results in defective kinetochore function and abnormal chromosome segregation (Castillo et al., 2007). This indicates that some kinetochore or centromere components may recognize the particular chromatin state formed after DNA replication. In agreement, the recently identified CENP-T/CENP-W complex was reported to specifically interact with H3 containing nucleosomes, but not with CENP-A nucleosomes within centromeric chromatin (Hori et al., 2008; Ribeiro et al., 2010). However, this view was nuanced by the recent finding that CENP-T/CENP-W can contact DNA directly (Nishino et al. 2012). Nevertheless, centromere recruitment of the CENP-T/CENP-W complex occurs during late S phase and G2, following replication of centromeric DNA, redistribution of the parental CENP-A nucleosomes and assembly of H3.1 and H3.3 into centromeric chromatin (Dunleavy et al., 2011; Prendergast et al., 2011). This suggests that the composition of centromeric chromatin achieved after DNA replication is a prerequisite for centromere targeting of CENP-T and CENP-W, which in turn are essential to form a functional kinetochore (Hori et al., 2008; Gascoigne et al., 2011). In summary, the mixed CENP-A/H3 chromatin state formed upon DNA replication may be critical for correct formation of the kinetochore and for proper segregation of chromosomes. The maintenance of such mixed chromatin state until mitosis is completed, can only be achieved by delaying assembly of CENP-A until mitotic exit. This provides a likely explanation for the unique timing of centromere propagation. An important test for this model will be determining whether assembly of CENP-A prior to mitosis causes kinetochore failure and errors in chromosome segregation.

Cdk activity and cell cycle control of CENP-A assembly in other organisms

The work presented in this thesis showed that CENP-A assembly in human and chicken DT40 cells occurs upon mitotic exit and requires loss of Cdk1 and Cdk2 activities (Figure 5.2 A). The timing of CENP-A assembly appears to be evolutionarily conserved in many eukaryotes, for example in *Drosophila* syncytial embryos and in meiotically arrested *Xenopus* egg extracts (Schuh et al., 2007; Bernad et al., 2011; Moree et al., 2011). Likewise, Cdks and their functions are also highly similar among eukaryotes. Here, I will speculate how Cdk activity controls the timing of CENP-A assembly in different organisms (Figure 5.2).

CENP-A^{Cnp1} assembly in *Schizosaccharomyces pombe*

In the fission yeast *Schizosaccharomyces pombe*, CENP-A^{Cnp1} is incorporated into centromeric chromatin during S and G2 phases (Takahashi et al., 2005; Takayama et al., 2008). However, the primary pathway of centromere propagation appears to occur during S phase (Takayama et al., 2008). In this organism, G1 phase is essentially nonexistent and S phase takes place shortly after mitotic exit and before cytokinesis. Therefore, as in vertebrate cells, initiation of CENP-A^{Cnp1} assembly follows completion of mitosis in fission yeast. However, in contrast to human and chicken cells, CENP-A^{Cnp1} incorporation into centromeric chromatin coincides with DNA replication and can also occur during G2 phase in a small subset of wild type cells (Takayama et al., 2008).

Despite these differences, the proteins required for CENP-A^{Cnp1} assembly appear to be highly conserved. As in vertebrates, CENP-A^{Cnp1} deposition in fission yeast also requires centromere localization of Mis18 and Scm3 (Figure 5.2 C), the fission yeast homologues of Mis18α/β and HJURP, respectively (Hayashi et al., 2004; Pidoux et al., 2009; Williams et al., 2009). In human cells, these proteins are targeted to the centromere only during anaphase and late telophase,

respectively (Fujita et al., 2007; Maddox et al., 2007; Dunleavy et al., 2009; Foltz et al., 2009; Silva and Jansen, 2009). In fission yeast, Mis18 and Scm3 associate with centromeres from anaphase through S and G2 phases and are released in early mitosis, when Cdk^{Cdc2} activity peaks [Figure 5.2 C, (Hayashi et al., 2004; Pidoux et al., 2009; Williams et al., 2009)]. Thus, I hypothesize that Cdk activity also plays a role in controlling the centromeric localization of these proteins in fission yeast (Figure 5.2 C). Consistent with this hypothesis, Scm3 is known to be phosphorylated *in vivo* and several putative Cdk phosphorylation sites are present in both Mis18 and Scm3 (Pidoux et al., 2009).

The fission yeast cell cycle is controlled by a single Cdk, Cdc2, and by four cyclins Cig1 and Puc1, which have minor roles in G1 phase, Cig2 that initiates DNA replication and Cdc13 that is required for mitosis (Stern and Nurse, 1996). I propose a model in which Cdc2-Cdc13 activity during mitosis prevents centromere targeting of Mis18 and Scm3, and consequently inhibits assembly of CENP-A^{Cnp1}. Loss of Cdc2-Cdc13 upon mitotic exit promotes the centromere localization of these proteins and assembly of CENP-A^{Cnp1}. However, Cdc2-Cdc13 is also active during G2 phase, when Mis18 and Scm3 are present at centromeres. In the absence of other cyclins, the oscillations of Cdc2-Cdc13 activity are sufficient to sequentially trigger DNA replication and mitosis (Coudreuse and Nurse, 2010). Based on this observation, Nurse and colleagues proposed that Cdc2-Cdc13 allow orderly progression through these major cell cycle events due to its differential affinity for G1/S and mitotic substrates. They suggest that low Cdc2-Cdc13 activity is sufficient to phosphorylate high affinity substrates, driving S phase progression, while high Cdc2-Cdc13 activity is needed to phosphorylate low affinity substrates, allowing mitotic progression. In analogy, I propose that Mis18 and Scm3 are low affinity substrates that are only phosphorylated during mitosis, when Cdc2-Cdc13 reaches its maximum activity (Figure 5.2 C). However, while this mechanism may prevent CENP-A assembly during mitosis it may not be sufficient to prevent CENP-A^{Cnp1} loading in late G2 phase of the cell cycle. This is consistent with the observation that in a subset of

cells CENP-A^{Cnp1} assembly can occur during G2 phase when Mis18 and Scm3 are present at the centromere. However, since this represents a minority of cells it is likely that other proteins, besides Mis18 and/or Scm3, are targeted and inhibited by Cdc2 activity, or that there is an additional mechanism preventing centromere propagation prior to mitotic exit.

CENP-A^{CID} assembly in *Drosophila melanogaster*

In *Drosophila melanogaster* syncytial embryos, CENP-A^{CID} assembly occurs in anaphase and requires exit from mitosis (Schuh et al., 2007), similarly to what occur in human cells. Interestingly, the cell division cycle in this system is extremely short and gap phases are essentially nonexistent (Lee and Orr-Weaver, 2003). The rate at which CENP-A^{CID} is loaded is sped up accordingly and is completed within minutes rather than hours as is observed in human cells. This suggests that centromeric chromatin assembly is not an inherently slow process but can be accelerated similar to what is observed for DNA replication.

In contrast to embryos, in *D. melanogaster* S2 and Kc167 cells CENP-A^{CID} assembly occurs during metaphase (Mellone et al., 2011), indicating that in these cells mitotic exit is not required. Indeed, arresting S2 cells in mitosis by microtubule poison does not prevent assembly (Mellone et al., 2011). While these observations appear at odds with our model that argues for Cdk-mediated inhibition of CENP-A assembly, they do not necessarily call for an entirely different model to explain cell cycle control in this system. Instead, I propose that, in these cells, CENP-A^{CID} assembly is held in check entirely by Cdk-cyclin A activity during S and G2 phases (Figure 5.2 B). The APC/C^{Cdc20}-mediated degradation of cyclin A in early mitosis (Geley et al., 2001; den Elzen and Pines, 2001) potentially enables assembly of CENP-A^{CID} into centromeres in these cells. Although at first glance the cell cycle regulation of CENP-A appears to be different between *D. melanogaster* cell lines and syncytial embryos they may, in fact, be similarly controlled by Cdk-cyclin A but cyclin A degradation may be

differentially regulated in these two cell types. In agreement with this hypothesis, in *D. melanogaster* syncytial embryos, cyclin B is only partially degraded upon mitotic exit and is not essential for mitosis (Knoblich and Lehner, 1993; Raff et al., 2002). Cyclin B is therefore an unlikely candidate to play a role in controlling the timing of centromere propagation. In contrast, cyclin A is essential for mitosis and its levels are maintained until anaphase during the initial mitotic divisions of the *D. melanogaster* syncytial embryo (Knoblich and Lehner, 1993). Taken together, based on these results, I suggest that in both *D. melanogaster* embryos as well as *D. melanogaster* cell lines, timing of CENP-A^{CID} assembly is controlled by Cdk-cyclin A activity.

In *D. melanogaster*, no homologues of the Mis18 complex or HJURP have been identified. Instead, CENP-A^{CID} assembly requires CENP-C and CAL1 (chromosome alignment defect 1) (Goshima et al., 2007; Erhardt et al., 2008). In S2 cells, CAL1 is recruited to centromeres in prophase, before CENP-A^{CID} (Figure 5.2 B), similar to the recruitment of the human Mis18 complex prior to CENP-A assembly in mammalian cells (Silva and Jansen, 2009; Mellone et al., 2011). Additionally, CAL1 associates with prenucleosomal CENP-A^{CID} and is required to stabilize CENP-A^{CID} protein levels, suggesting that CAL1 could function as a CENP-A^{CID} specific chaperone (Mellone et al., 2011). In flies, CAL1 appears to combine the functions of the Mis18 complex and HJURP. This suggests that the timing of CENP-A^{CID} assembly in flies may be regulated through Cdk-cyclin A-mediated phosphorylation of CAL1 (Figure 5.2 B). Indeed, CAL1 harbors several putative Cdk phosphorylation sites.

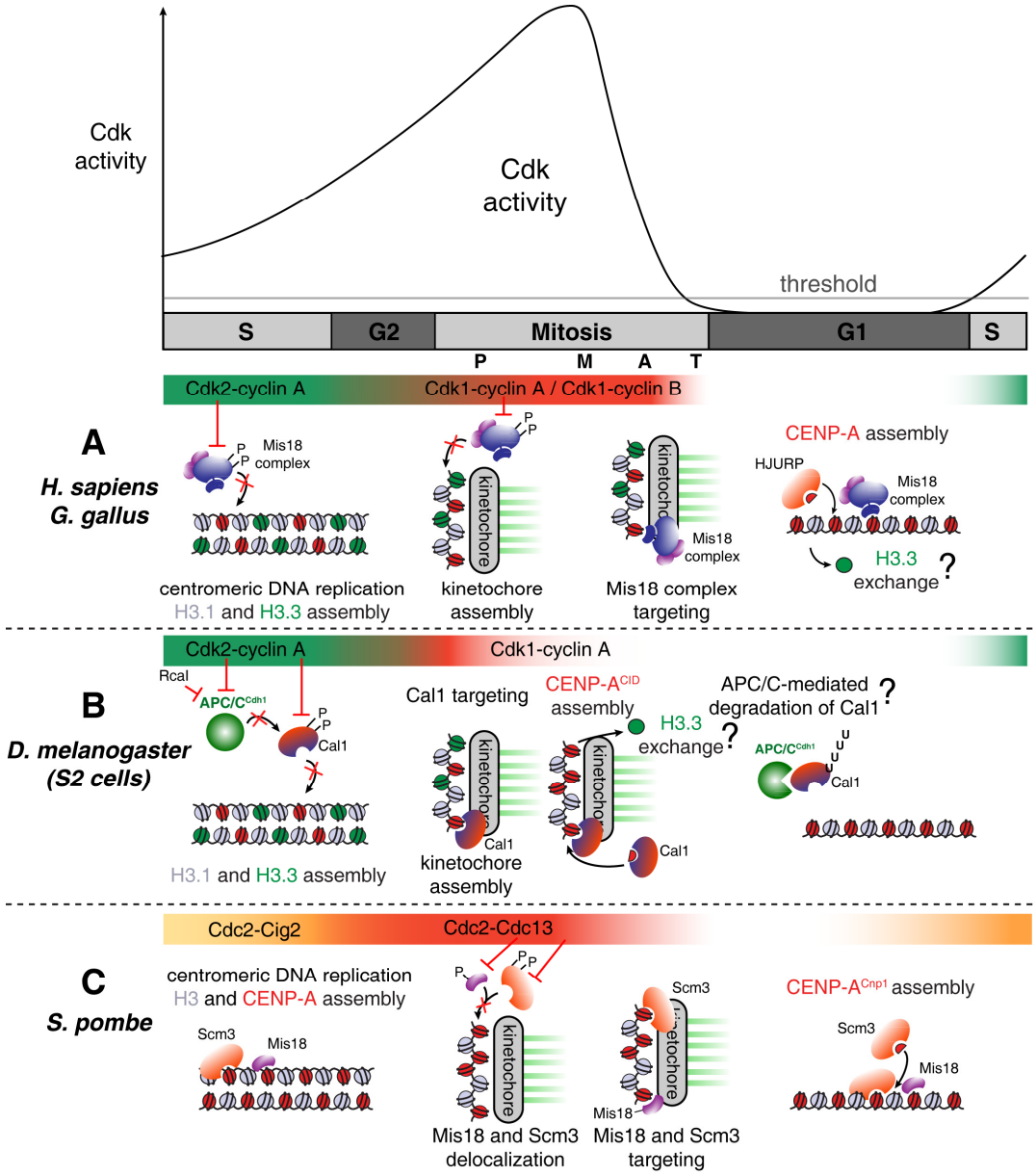

Figure 5.2. Speculative models depicting how Cdk activity controls the timing of CENP-A assembly in different organisms. (A) In human and DT40 cells, CENP-A assembly is inhibited throughout S, G2, and mitosis by Cdk1/2-cyclin A and Cdk1-cyclin B activities. This inhibition likely involves Cdk-mediated phosphorylation of Mis18BP1^{HsKNL2}, which prevents targeting of the Mis18 complex to centromeres and HJURP recruitment. Continued on the next page.

Figure 5.2. (continued) APC/C-mediated degradation of cyclins A and B leads to loss of Cdk1 and Cdk2 activities during mitotic exit, which alleviates the inhibitory phosphorylation of Mis18BP1^{HsKNL2}. This allows targeting of the Mis18 complex during anaphase, followed by HJURP recruitment and CENP-A assembly in late telophase/early G1. (B) In S2 cells, CENP-A^{CID} assembly is inhibited throughout S and G2 by Cdk1/2-cyclin A activities. Cdk1/2-cyclin A phosphorylates Cal, which keeps it in an inactive, noncentromeric state. At the onset of mitosis, cyclin A is degraded in an APC/C^{CDC20} dependent manner, leading to inactivation of Cdk1/2-cyclin A. This alleviates the inhibitory phosphorylation of Cal1, and allows its targeting to centromeres in prophase, followed by CENP-A assembly in metaphase. Upon anaphase onset, APC/C^{Cdh1} activation leads to destruction of Cal1, shutting down the CENP-A assembly machinery. (C) In fission yeast, CENP-A^{Cnp1} assembly is held in check during G2 and mitosis through action of Cdc2-Cdc13 activity. Centromere propagation in this system involves Mis18 and Scm3, which are localized at centromeres throughout the cell cycle, except during early mitosis. In early mitosis, Cdc2-Cdc13 phosphorylates Mis18 and Scm3, preventing their centromere targeting. Loss of Cdc2 activity upon mitotic exit allows relocalization of Mis18 and Scm3 to centromeres in anaphase, followed by assembly of CENP-A^{Cnp1} into centromeric chromatin during S phase (Note that in this system G1 phase is nearly inexistent).

Intriguingly, depletion of cyclin A causes a defect in CENP-A^{CID} targeting to centromeres (Erhardt et al., 2008). RCA1 (fly homologue of mammalian Emi1) was also shown to be required for CENP-A^{CID} assembly in S2 cells (Erhardt et al., 2008). Both cyclin A and RCA1 play important roles during cell cycle progression: while cyclin A activates Cdk1 and Cdk2, RCA1 prevents APC/C^{Cdh1} activation during G2 phase (Dong et al., 1997; Morgan, 1997). Cdk2-cyclin A activity is also able to inhibit APC/C^{Cdh1} (Lukas et al., 1999). Therefore a common feature of both cyclin A and RCA1 is that they both block activation of APC/C^{Cdh1}, and that premature activation of APC/C^{Cdh1} is deleterious for CENP-A^{CID} assembly. Consistent with this notion, depletion of Cdh1 rescues the defect in CENP-A^{CID} assembly caused by cyclin A and RCA1 depletion (Erhardt et al., 2008). CAL1 levels at centromeres decline between metaphase and late anaphase when APC/C^{Cdh1} becomes active (Erhardt et al., 2008), suggesting CAL1 may be a substrate of the APC/C^{Cdh1} (Figure 5.2 B).

In summary, I propose a model that, although unproven, is consistent with the available evidence. CAL1 may be inhibited by Cdk-cyclin A-dependent phosphorylation and also be subject to destruction through APC/C^{Cdh1} activity. Inhibition of APC/^{Cdh1} by RCA1 and Cdk2-cyclin A allows accumulation of CAL1 during G2 phase which is kept in a noncentromeric state by Cdk-cyclin A-dependent phosphorylation. Degradation of cyclin A by APC/C^{Cdc20} in early mitosis will alleviate the inhibitory state of CAL1, targeting this protein to centromeres in prophase triggering CENP-A^{CID} assembly in metaphase. Subsequent activation of APC/C^{Cdh1} may lead of destruction of CAL1, restricting CENP-A^{CID} assembly to a narrow cell cycle window (Figure 5.2 B).

CENP-A^{CENH3} assembly in *Arabidopsis thaliana*

Unlike vertebrates, plants appear to assemble CENP-A^{CENH3} into centromeric chromatin before mitosis (Lermontova et al., 2006, 2007, 2011). Centromeric CENP-A^{CENH3} levels in *Arabidopsis thaliana* are highest in G2 phase cells, suggesting assembly in this phase of the cell cycle. FRAP analysis of YFP-CENP-A^{CENH3} showed little turnover outside of G2 phase (Lermontova et al., 2011). However, assembly in G2 phase was not confirmed with this technique. It is possible that a thus far unknown CENP-A^{CENH3}-specific assembly factor is expressed and/or activated only during G2 phase. Plants and vertebrates differ not only in the timing of CENP-A deposition at centromeres, but also in the CENP-A assembly factors involved. In plants, proteins required for CENP-A^{CENH3} assembly or homologues of the known CENP-A assembly factors from other organisms have not been identified (Lermontova et al., 2011). Taken together, these results suggest that, while in plants CENP-A assembly and bulk chromatin assembly are also temporally separated, the mechanism through which this is achieved is not shared with metazoans and yeast.

Although in plants Cdk activity does not appear to play a role in controlling the timing of CENP-A^{CENH3} assembly, it was recently shown to play a role in

regulating CENP-A^{CENH3} transcription (Heckmann et al., 2011). In *Arabidopsis thaliana, CENP-A* expression is regulated by members of the E2F family of transcription factors in a cell cycle dependent manner (Heckmann et al., 2011). The activity of E2F transcription factors is necessary for the transcriptional activation of many genes required for S phase progression. Alternatively, these genes can be repressed through inhibition of E2F, which is achieved by E2F association with members of the Rb protein family (Harbour and Dean, 2000; Singh et al., 2010). Phosphorylation of Rb proteins by Cdk4/6-cyclin D and Cdk2-cyclin E releases Rb from the E2F transcription factors, resulting in transcription activation of E2F-target genes, including *CENP-A* (Lundberg and Weinberg, 1998). In summary, in *A. thaliana*, Cdk activity appears to positively regulate centromere propagation, through activation of *CENP-A* gene transcription. Whether timed transcription of the CENP-A gene contributes to cell cycle restricted assembly of CENP-A chromatin has not been determined.

Cdks control CENP-A assembly at two distinct levels

The E2F pathway is highly conserved in higher eukaryotes (Inzé, 2005). *In silico* analysis revealed a putative E2F binding site in the promoter of human CENP-A and at least two E2F binding sites in the promoter of *D. melanogaster* CENP-A^{CID}, suggesting that the E2F-mediated transcriptional regulation of CENP-A is evolutionary conserved (Lermontova et al., 2011). In agreement with this notion, in human cells, reduction of Rb levels result in an increase in CENP-A transcript and protein levels (Sullivan et al., 2011).

In summary, at least in humans, Cdks may regulate CENP-A assembly at two distinct levels, while rising Cdk activity in early S phase induces *CENP-A* transcription and inhibits CENP-A assembly machinery, loss of Cdk activity activates CENP-A assembly machinery and down regulates *CENP-A* transcription. This dual regulation may contribute to restrict CENP-A chromatin

size by preventing CENP-A expression during CENP-A chromatin assembly. Although Cdk activity appears to regulate centromere propagation both positively and negatively, Cdk-mediated inhibition appears to be dominant in cell cycle control because forcing CENP-A expression throughout the cell cycle using a CMV or other viral promoter does not change the timing of CENP-A assembly.

General importance of the principles of centromere inheritance

Since centromeres are crucial for proper cell division, a thorough understanding of centromere propagation and its regulation may be of key importance for our understanding of tumor development. Defective centromere function leads to chromosome segregation errors and formation of aneuploid cells, which potentially leads to the development of cancer (Yoda and Tomonaga, 2004; Weaver et al., 2007). Consistently, overexpression of CENP-A and CENP-H was found in colorectal cancers (Tomonaga et al., 2003, 2005). Moreover, neocentromeres, which can be formed through loss or repositioning of the original centromere, has been observed in some tumor categories such as lipomatous tumors, acute myeloid leukemia and lung carcinoma (reviewed in Marshall et al., 2008).

The complete dissection of the mechanisms responsible for the epigenetic inheritance of the centromere will also have crucial implications for our knowledge of general principles of epigenetic inheritance that are fundamental to transcription regulation, genome organization and pathology, when they are defective (Holliday, 2006; Feinberg, 2007; Iacobuzio-Donahue, 2009; Black and Whetstine, 2011).

Cdk1 and Cdk2 as general regulators of epigenetic inheritance throughout cell divisions

The work described in this thesis demonstrates that Cdk activity couples epigenetic centromere inheritance to cell cycle progression. Using the centromere as a model and taking advantage of the SNAP-based pulse labeling technique, we revealed a key mechanism of how epigenetic marks can be maintained and replenished across cell divisions. Concurrently with our work, it was shown that Cdk1 and Cdk2 phosphorylate the enzymatic subunit EZH2 of the polycomb repressive complex 2 (PRC2) (Chen et al., 2010). EZH2 catalyzes trimethylation of Lysine 27 of histone H3 (H3K27me3) (Cao et al., 2002; Kuzmichev et al., 2002) and plays a essential role in epigenetic gene silencing, which is important for X-chromosome inactivation, developmental patterning and maintenance of stem cell pluripotency [reviewed in Chapter1, Section 3.1.3; (Plath et al., 2003; Lee et al., 2006; Shen et al., 2009)]. Cdk-mediated phosphorylation of EZH2 is important for its recruitment to target loci and for maintenance of H3K27me3 at those sites (Chen et al., 2010). Thus, Cdk1 and Cdk2 activities ensure that the H3K27me3 epigenetic mark is copied into nascent H3 containing nucleosomes during S and G2 phases, allowing propagation of this epigenetic mark across cell divisions.

DNA methylation also plays a crucial role in the epigenetic regulation of gene expression and this epigenetic mark is inherited and maintained through action of the DNA methyltransferase 1 (DNMT1) (reviewed in Chapter1, Section 3.1.1). Recently, it was shown that Cdk1, Cdk2 and Cdk5 phosphorylate DNMT1, which promotes its enzymatic activity and its stability (Lavoie and St-Pierre, 2011). These results show that Cdk activity is important for maintenance of DNA methylation patterns in cycling cells, reinforcing the importance of Cdks in epigenetic inheritance.

In summary, Cdk activity plays an important role in coupling the inheritance of distinct epigenetic marks with cell division. In some cases, Cdk activity

positively regulates the propagation of epigenetic marks such as DNA methylation and H3K27me3, whereas in others, Cdk activity can negatively regulate their propagation such as in the case of CENP-A assembly. In either case, tight coupling between Cdk activity and inheritance of epigenetic marks may be important to ensure that those marks are propagated only once per cell cycle, avoiding their loss or over accumulation.

Future prospects for the study of centromere inheritance

In this thesis I have discussed novel evidence on the molecular mechanisms that couple epigenetic centromere inheritance with cell cycle progression. We show that the only mitotic signal required for triggering CENP-A assembly is loss of Cdk1 and Cdk2 activity upon mitotic exit. We also demonstrate that centromere targeting of Mis18BP1^{HsKNL2} depends on its phosphorylation status. Further experiments are required to test whether this protein is directly phosphorylated by Cdk1 or Cdk2. The premature centromere targeting of a Mis18BP1^{HsKNL2} mutant that can no longer be phosphorylated is not sufficient to induce CENP-A assembly prior to mitosis, indicating that other proteins are phosphorylated and inhibited by Cdks. The identification of these additional players is required to firmly establish the molecular mechanisms through which Cdk1 and Cdk2 prevent centromere propagation until completion of mitosis.

One important issue raised by the results presented in this thesis is to understand why S phase cells are less permissive for CENP-A assembly. Future efforts should be focused on the regulation of CENP-A assembly by additional mechanisms during DNA replication. Future work should also explore the biological short term consequences of assembling CENP-A nucleosomes prior to mitosis. It would be important to test whether the kinetochore is formed properly

and is able to bind efficiently to spindle microtubules when faced with an imbalance in the centromeric chromatin.

Importantly, it has been reported that one tenth of the normal CENP-A level at the centromere is enough to support functional kinetochore assembly and proper chromosome segregation (Liu et al., 2006), suggesting that there is some plasticity in the composition of centromeric chromatin. In light of this it would be interesting to test if centromeric chromatin can also support more CENP-A nucleosomes than its normal mitotic complement. A related question is whether cells following induced assembly in G2 phase retain the capacity to reload in the subsequent G1 phase. If so, live cell analysis could be used to determine the long term consequences of overloading centromeric chromatin with CENP-A nucleosomes.

In the future it would also be important to integrate the knowledge coming from different model organisms to fully understand the molecular basis of the epigenetic and cell cycle regulation of centromere propagation. Despite some of the differences in centromere propagation among eukaryotes, the timing and the proteins involved in CENP-A assembly appear to be evolutionarily conserved. Based on the hypothesis we propose above that explain how centromere propagation occurs in flies (Figure 5.2 B), it would be interesting to explore the role of APC/C^{Cdh1} in degradation of proteins required for CENP-A assembly in human cells. How CENP-A assembly is turned off is still very poorly understood. It is possible that similar to CAL1, members of the Mis18 complex or HJURP are a target for APC/C^{Cdh1}-mediated degradation during the G1 phase of the cell cycle.

Finally, to fully understand how centromeres are epigenetically inherited across generations it is important to go beyond our understanding of how new CENP-A nucleosomes are incorporated into centromeric chromatin. It is equally critical to understand how parental CENP-A nucleosomes are maintained and reassembled into chromatin after DNA replication.

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Appendix 1 – Deletion mutants of Mis18α, Mis18β and HJURP and effect on CENP-A assembly

Appendix 1 – Deletion mutants of Mis18α, Mis18β and HJURP and effect on CENP-A assembly

Table A1. List of primers used for strand exchange PCR		
Mutant protein	Primer name	Primer sequence
HJURP-Ala^{8}-GFP	CenCAF_5KpnI	CTTGGTACCGCCACCATGC
	CenCAF_3TS122-3AA_S128A	GCCTCTTCCTGGTCTGCCGCGGCATCGACCTCACCGCTTTTTG
	CenCAF_5TS122-3AA_S128A	CGGCAGACCAGGAAGAGGCAGTTGCTTGGGC
	CenCAF_3S140A	CATTTTTCAAAGGGGCTTGAGGCACTGC
	CenCAF_5S140A	GCAGTGCCTCAAGCCCCTTTGAAAAATG
	CenCAF_3S448A	CTGGTTCCTGGGAGCCAGGCAATATTC
	CenCAF_5S448A	GAATATTGCCTGGCTCCCAGGAACCAG
	CenCAF_3S473A	GAAGGCCACCAGGAGCCGCAGGACCCCCTCTG
	CenCAF_5S473A	CAGAGGGGGTCCTGCGGCTCCTGGTGGCCTTC
	CenCAF_3S642A	CCCCAGGGGTGCTGATGGCAACTTC
	CenCAF_5S642A	GAAGTTGCCATCAGCACCCCTGGGG
	CenCAF-3EcoRI	GCAGAATTCCCCACACTTTTAGTTTC
HJURP$^{\Delta N51}$-GFP	CenCAF_5KpnIdelN51	CATGGTACCACCATGGCCACGCTGACCTACG
	CenCAF-3EcoRI	GCAGAATTCCCCACACTTTTAGTTTC
HJURP$^{\Delta 51\text{-}183}$-GFP	CenCAF_5KpnI	CTTGGTACCGCCACCATGC
	CenCAF_3del52-183	GCAGGCACGGCAGGTGAGGCTTGCACCACCGGGGTGTC
	CenCAF_5del52-183	GCCTCACCTGCCGTGCC
	CenCAF-3EcoRI	GCAGAATTCCCCACACTTTTAGTTTC
HJURP$^{\Delta 184\text{-}282}$-GFP	CenCAF_5KpnI	CTTGGTACCGCCACCATGC
	CenCAF_3del184-282	TGCATGATGAACGTTTTGGTGGAGATCAGTGAAGGCAGCGGAGTC
	CenCAF_5del184-282	ATCTCCACCAAAACGTTCATCATG
	CenCAF-3EcoRI	GCAGAATTCCCCACACTTTTAGTTTC

Appendix 1 – Deletion mutants of Mis18α, Mis18β and HJURP and effect on CENP-A assembly

Table A1. Continued		
Mutant protein	Primer name	Primer sequence
HJURP$^{\Delta 283-487}$-GFP	CenCAF_5KpnI	CTTGGTACCGCCACCATGC
	CenCAF_3del283-487	CTTTTTGCTTTTGCTTTGCTGGAAGGGATGCTTGATGGCTTTGTGC
	CenCAF_5del283-487	CCTTCCAGCAAAGCAAAAGC
	CenCAF-3EcoRI	GCAGAATTCCCCACACTTTTAGTTTC
HJURP$^{\Delta 488-640}$-GFP	CenCAF_5KpnI	CTTGGTACCGCCACCATGC
	CenCAF_3del488-640	TTCTGCACCCCAGGGGTGATGATAAACTCAGCCTGCGGGTTTC
	CenCAF_5del488-640	TCATCACCCCTGGGGTGC
	CenCAF-3EcoRI	GCAGAATTCCCCACACTTTTAGTTTC
HJURP$^{\Delta C640}$-GFP	CenCAF_5KpnI	CTTGGTACCGCCACCATGC
	CenCAF_3EcoRIdelC640	CATGAATTCCCTGGCAACTTCTGGAAACCCTG
GFP-Mis18α$^{\Delta N75}$	Mis18a^N75for	GAGAATTCTCGCGGAGGAGAGGCCGCTG
	3BamHI_Mis18a	GATTTTTTCAGTGCAGCACCTTTACAGCATC
GFP-Mis18α$^{\Delta 100-129}$	Mis18a_5EcoRI	GAGAATTCTCGCAGGCGTTCGGTCACTGAG
	Mis18a^100-129rev	CACAAAGTCTCAAGGACGCAACCATTTTCCTCCTGGCTGGCCACCCAG
	Mis18a^100-129for	GAAAATGGTTGCGTCCTTGAGAC
	3BamHI_Mis18a	GATTTTTTCAGTGCAGCACCTTTACAGCATC
GFP-Mis18α$^{\Delta C157}$	Mis18a_5EcoRI	GAGAATTCTCGCAGGCGTTCGGTCACTGAG
	Mis18a^C151rev	GAGGATCCTCAGTAGCCAAGATTGAGTGAGCACCC
GFP-Mis18β$^{\Delta N69}$	Mis18B-5XhoI_N69	CATCTCGAGCTGCAGCCTGAGAGGTGCGC
	Mis18B-3EcoRI	CATGAATTCTCAGTTTTCTGGCTTGGACTGGTCAG

Appendix 1 – Deletion mutants of Mis18α, Mis18β and HJURP and effect on CENP-A assembly

Table A1. Continued		
Mutant protein	Primer name	Primer sequence
GFP-Mis18β$^{\Delta 100\text{-}126}$	Mis18B-5XhoI	CATCTCGAGGCGGCTCAGCCGCTGCG
	Mis18B_3del100-126	CACAGAATAAAAGGTTGTAAGTACTGCCTTTGAGCCGCGACAGGTCCCAGG
	Mis18B-5del100-126	CTCAAAGGCAGTACTTACAACCTTTTATTC
	Mis18B-3EcoRI	CATGAATTCTCAGTTTTCTGGCTTGGACTGGTCAG
GFP-Mis18β$^{\Delta 156\text{-}184}$	Mis18B-5XhoI	CATCTCGAGGCGGCTCAGCCGCTGCG
	Mis18B-3del156-184	ATCTTTTCTGATAGAGGAACATTTTGAATATCCATCAGGGCAGCATGGGTAGAATAC
	Mis18B-5del156-184	ATGGATATTCAAAATGTTCCTCTATCAG
	Mis18B-3EcoRI	CATGAATTCTCAGTTTTCTGGCTTGGACTGGTCAG
GFP-Mis18β$^{\Delta C185}$	Mis18B-5XhoI	CATCTCGAGGCGGCTCAGCCGCTGCG
	Mis18B-3EcoRI_C185	CATGAATTCTCACTCTGATGCATTTACTATGGCTTTTG

Appendix 1 – Deletion mutants of Mis18α, Mis18β and HJURP and effect on CENP-A assembly

Table A2. Deletion mutants of Mis18α, Mis18β and HJURP and effect on CENP-A assembly

Protein	Localization of the mutant protein	CENP-A localization in G1 phase	CENP-A localization in G2 phase
GFP-Mis18α WT	cytoplasmic and nuclear in G2 and centromeric from late anaphase to mid G1	centromeric	nucleolar
GFP-Mis18α$^{\Delta N75}$	cytoplasmic and nuclear throughout cell cycle	centromeric	nucleolar
GFP-Mis18α$^{\Delta 100\text{-}129}$	cytoplasmic and nuclear throughout cell cycle	centromeric	nucleolar
GFP-Mis18α$^{\Delta C157}$	cytoplasmic and nuclear throughout cell cycle	centromeric	nucleolar
GFP-Mis18β WT	cytoplasmic and nuclear in G2 and centromeric from late anaphase to mid G1	centromeric	nucleolar
GFP-Mis18β$^{\Delta N69}$	cytoplasmic and nuclear throughout cell cycle	centromeric	nucleolar
GFP-Mis18β$^{\Delta 100\text{-}126}$	cytoplasmic and nuclear throughout cell cycle	centromeric	nucleolar
GFP-Mis18β$^{\Delta 156\text{-}184}$	cytoplasmic and nuclear throughout cell cycle	centromeric	nucleolar
GFP-Mis18β$^{\Delta C185}$	cytoplasmic and nuclear in G2 and centromeric from late anaphase to mid G1 (few G2 with centromeric signal)	centromeric	nucleolar
HJURP WT-GFP	nuclear or nucleolar in G2 and centromeric in late telophase/early G1	centromeric	nucleolar
HJURP$^{\Delta N51}$-GFP	nuclear throughout cell cycle	centromeric	nucleolar
HJURP$^{\Delta 51\text{-}183}$-GFP	nuclear throughout cell cycle	centromeric	nucleolar

Appendix 1 – Deletion mutants of Mis18α, Mis18β and HJURP and effect on CENP-A assembly

Table A2. Continued			
Protein	Localization of the mutant protein	CENP-A localization in G1 phase	CENP-A localization in G2 phase
HJURP$^{\Delta 184-282}$-GFP	nuclear or nucleolar in G2 and centromeric in late telophase/early G1 and in some G2 cells	centromeric	centromeric (in a small percentage of G2 cells)
HJURP$^{\Delta 283-487}$-GFP	nuclear or nucleolar in G2 and nuclear aglomerates in late telophase/early G1	centromeric	nucleolar
HJURP$^{\Delta 488-640}$-GFP	nuclear or nucleolar throughout cell cycle	centromeric	nucleolar
HJURP$^{\Delta C640}$-GFP	nuclear or nucleolar in G2 and centromeric in late telophase/early G1	centromeric	nucleolar

Appendix 2 – Centromere assembly requires the direct recognition of CENP-A nucleosomes by CENP-N

LETTERS

nature cell biology

Centromere assembly requires the direct recognition of CENP-A nucleosomes by CENP-N

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Centromeres are specialized chromosomal domains that direct kinetochore assembly during mitosis. CENP-A (centromere protein A), a histone H3-variant present exclusively in centromeric nucleosomes, is thought to function as an epigenetic mark that specifies centromere identity. Here we identify the essential centromere protein CENP-N as the first protein to selectively bind CENP-A nucleosomes but not H3 nucleosomes. CENP-N bound CENP-A nucleosomes in a DNA sequence-independent manner, but did not bind soluble CENP-A–H4 tetramers. Mutations in CENP-N that reduced its affinity for CENP-A nucleosomes caused defects in CENP-N localization and had dominant effects on the recruitment of CENP-H, CENP-I and CENP-K to centromeres. Depletion of CENP-N using siRNA (short interfering RNA) led to similar centromere assembly defects and resulted in reduced assembly of nascent CENP-A into centromeric chromatin. These data suggest that CENP-N interprets the information encoded within CENP-A nucleosomes and recruits other proteins to centromeric chromatin that are required for centromere function and propagation.

Accurate chromosome segregation during mitosis is essential for the maintenance of genome integrity. Eukaryotic cells have evolved complex machinery to ensure the fidelity of chromosome segregation. Each chromosome directs the assembly of a kinetochore that mediates attachment to the mitotic spindle and is required for microtubule-dependent chromosome movement during mitosis. Kinetochores also function as signalling centres for the mitotic checkpoint, which delays the initiation of anaphase in response to improper chromosome attachment to the mitotic spindle (see ref. 1 for review). The centromere is the region of the chromosome on which the kinetochore is assembled. Human centromeric DNA is composed of repetitive α-satellite sequences but centromeres are thought to be epigenetically specified, as centromeric DNA is not well conserved between species and no DNA sequences have been identified that are necessary or sufficient for kinetochore function in vertebrates (see ref. 2 for review). In all eukaryotes, centromeric chromatin contains specialized nucleosomes in which histone H3 is replaced by a histone H3-variant, CENP-A (see ref. 3 for review). This centromere protein is currently the best candidate for the epigenetic mark that specifies centromere identity. It is essential for the centromeric recruitment of most other proteins required for kinetochore function, but the molecular basis for recognition of CENP-A-containing chromatin as the site of kinetochore assembly is poorly understood. Furthermore, the mechanisms that target newly synthesized CENP-A/H4 to established centromeric chromatin to maintain centromere identity have not been determined.

A fundamental limitation in understanding centromere assembly is the lack of well-defined biochemical assays for studying this process. No direct and specific interaction between CENP-A nucleosomes and any of the > 75 proteins that make up a vertebrate mitotic kinetochore has been demonstrated. To identify CENP-A nucleosome-interacting proteins, we developed a simple and rapid binding assay using reconstituted mononucleosomes that contained α-satellite DNA derived from human centromeres and either histone H3 or CENP-A (Fig. 1a). Potential CENP-A nucleosome-interacting proteins were selected for testing in this assay based on the analysis of kinetochore assembly in several organisms. We focused on the constitutive centromere associated network (CCAN) of proteins, which includes the centromere proteins CENP-C, H, I and K–U, because these proteins are localized to centromeres during interphase and are required for the assembly of functional kinetochores in mitosis. Moreover, their localization, observed by immuno-electron microscopy of CENP-C, to the inner kinetochore plate and the recent demonstration that several of these proteins co-purify with CENP-A mononucleosomes suggests that CCAN proteins are likely to be the chromatin proximal elements of kinetochores[4–7]. We also included CENP-B in our analysis because it has been shown previously to bind directly to a conserved 17-nucleotide motif called the CENP-B box, present in α-satellite DNA[8]. We expressed CENP-B, C, H, I, and K–U and labelled them with ^{35}S by coupled *in vitro* transcription and translation (Fig. 1b; Supplementary Information Fig. S1). We then incubated the labelled proteins with or without CENP-A mononucleosomes before resolving the mixtures using native gel electrophoresis. Both CENP-B and CENP-N showed an increased relative migration in the presence of CENP-A nucleosomes assembled with α-satellite DNA, suggesting that CENP-B and CENP-N

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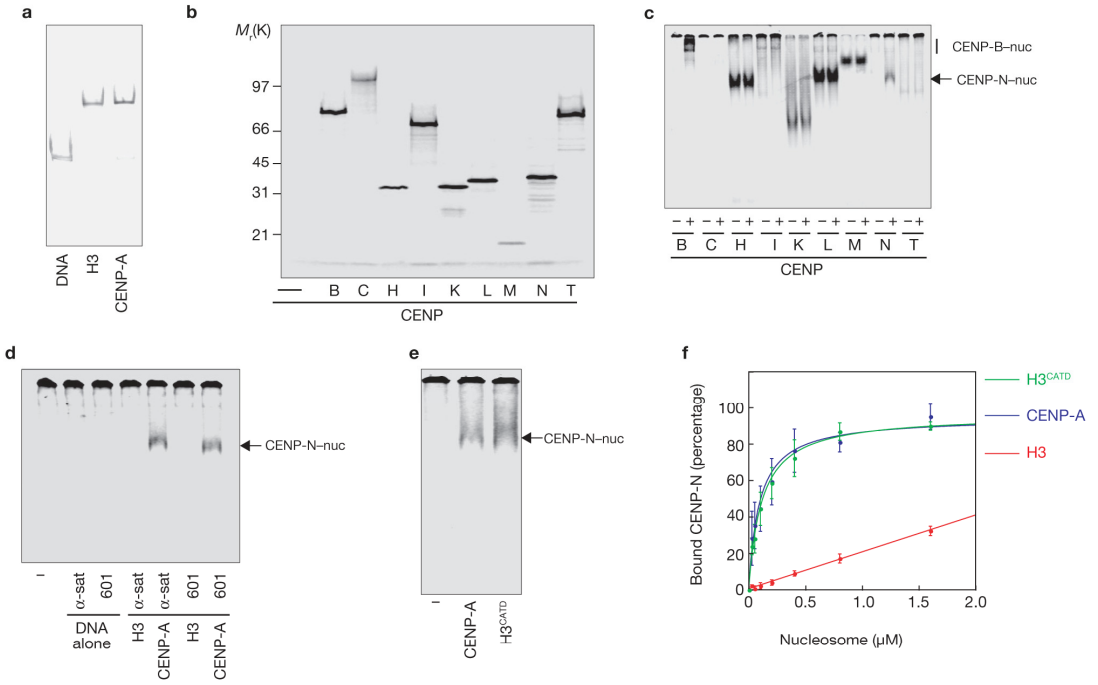

Figure 1 CENP-N binds CENP-A nucleosomes. (**a**) Reconstitution of conventional and centromeric nucleosomes. Free 186 base pair human α-satellite DNA (DNA) or mononucleosomes containing either histone H3 or CENP-A were assembled by salt dialysis and resolved by native gel electrophoresis. (**b**) *In vitro* expression of centromere proteins. Centromere proteins to be used as substrates in nucleosome binding assays were expressed in coupled transcription and translation reactions containing ^{35}S-methionine. (**c**) CENP-A nucleosome binding assay. ^{35}S-labelled centromere proteins (~1 nM) were incubated in the presence (+) or absence (-) of reconstituted CENP-A nucleosomes (50 nM) and separated by native gel electrophoresis. Both CENP-B and CENP-N showed CENP-A-nucleosome dependent changes in migration (CENP-B/N–nuc, nucleosome bound CENP proteins). (**d**) CENP-N binding to nucleosomes depends on CENP-A and not DNA sequence. α-satellite DNA (α-sat) or 601 DNA (601), or H3- or CENP-A-containing nucleosomes reconstituted with α-satellite or 601 DNA were incubated with ^{35}S-labelled CENP-N and resolved by native gel electrophoresis. The faster migrating band (arrow) indicates nucleosome-bound CENP-N (– indicates no nucleosome or DNA, as a control). (**e**) Binding of CENP-N to CENP-A nucleosomes occurs through the CATD region of CENP-A. No nucleosome (–) as a control, CENP-A or H3^{CATD} nucleosomes reconstituted with α-satellite DNA were bound to ^{35}S–CENP-N and assayed as in **c**. (**f**) CENP-N binds with equal affinity to CENP-A and H3^{CATD} nucleosomes. CENP-N binding was assayed as in **c** with increasing nucleosome concentration and was quantified according to the ^{35}S–CENP-N signal in the gel (data are mean ± s.e.m., $n = 3$).

bind directly to CENP-A nucleosomes (Fig. 1c). We did not detect any change in the migration of CENP-C, H, I, K, L, M or O–U (Fig. 1c; Supplementary Information, Fig. S1a). We also tested hMis18α, hMis18β and M18BP1/hKNL-2, which have been implicated in CENP-A assembly, but we did not detect any interaction with CENP-A nucleosomes in this assay (Supplementary Information, Fig. S1b)[9,10].

We determined the contribution of DNA sequence and histone protein composition to CENP-B and CENP-N binding of CENP-A nucleosomes by alternately exchanging the nucleosomal DNA and histones. As expected, CENP-B bound to naked α-satellite DNA as well as both H3 and CENP-A nucleosomes that contained α-satellite DNA (Supplementary Information, Fig. S1c). CENP-B did not bind the synthetically derived 601 nucleosome positioning sequence or nucleosomes that contained the 601 DNA sequence. In contrast, CENP-N bound equally well to CENP-A nucleosomes that contained either the α-satellite DNA or the 601 DNA sequence (Fig. 1d). CENP-N did not bind the α-satellite or 601 DNA fragment or histone H3 nucleosomes assembled on either DNA fragment. CENP-N also bound to CENP-A/H4 tetrasomes reconstituted with α-satellite DNA that lacked histone H2A and H2B, but did not specifically bind to soluble CENP-A/H4 tetramers (Supplementary Information, Fig. S1d–f). Thus, CENP-N binds specifically to CENP-A-associated chromatin.

Domain transfer experiments have previously suggested that a contiguous portion of the loop I and helix II region within the histone fold of CENP-A, called the CENP-A targeting domain (CATD), is sufficient for CENP-A function *in vivo*[11,12]. We tested whether the CATD domain was sufficient for CENP-N binding using reconstituted nucleosomes that contained the histone H3^{CATD} chimaera. CENP-N bound efficiently to H3^{CATD}-containing nucleosomes (Fig. 1e), and dose-response experiments revealed that the affinity of CENP-N for H3^{CATD} nucleosomes was indistinguishable from the affinity of CENP-N for wild-type CENP-A nucleosomes (apparent K_d = 163 nM ± 60 versus 169 nM ± 70, respectively; Fig. 1f; Supplementary Information, Table S1). The CATD domain imparts structural differences to CENP-A nucleosomes, in comparison with H3 nucleosomes, and CENP-A nucleosomes have been suggested to function as an epigenetic mark within chromatin to specify centromere identity[13]. Our data suggest that CENP-N recognizes the unique structural information encoded by the CATD within CENP-A nucleosomes.

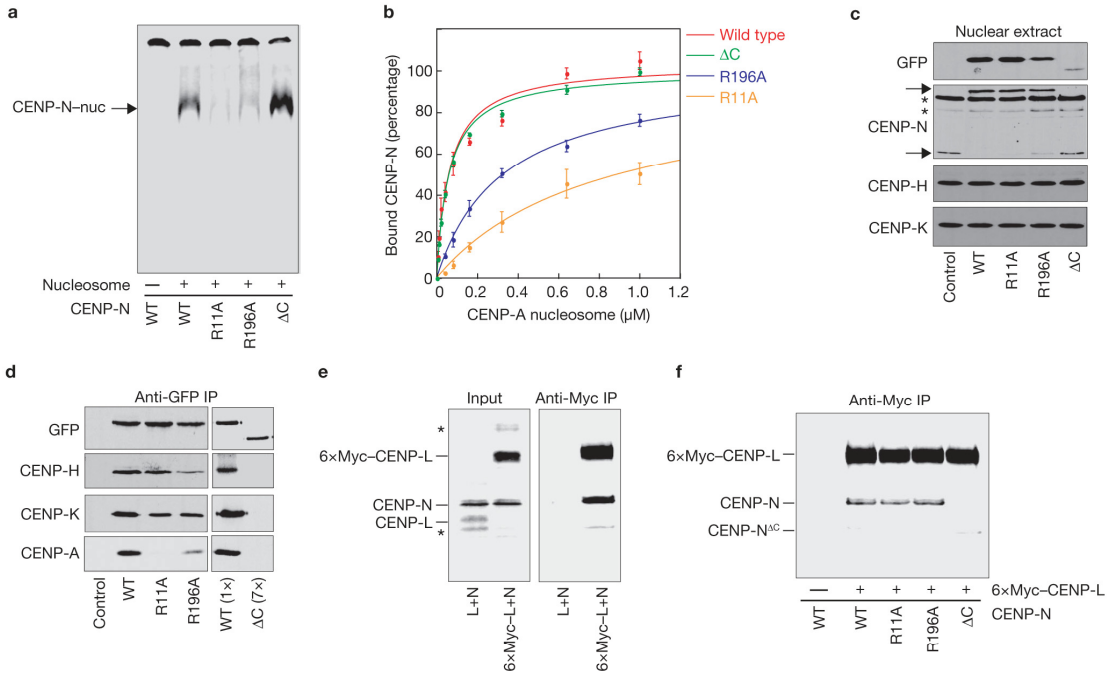

Figure 2 Identification and characterization of CENP-N mutants defective in CENP-A-nucleosome binding. (**a**) CENP-N mutants show a range of affinities for CENP-A nucleosomes. CENP-N wild type (WT) or the indicated CENP-N mutants (R11A, R196A and ΔC) were expressed and an equal concentration (~1 nM) of each was assayed for the ability to bind CENP-A nucleosomes (CENP-N–nuc, bound CENP). The control reaction (–) lacked CENP-A nucleosomes. (**b**) Dose-response experiments for each CENP-N mutant were performed as in **a**, with increasing concentrations of CENP-A nucleosome added to each reaction. Data are mean ± s.e.m., $n = 3$. (**c**) Expression of CENP-N mutants in HEK293 cells. Nuclear extracts from stable HEK293 cell lines expressing GFP–CENP-N (WT) or the indicated GFP–CENP-N mutants were separated by SDS–PAGE and analysed by western blotting using the indicated antibodies. Two nonspecific bands (asterisks) are present in the anti-CENP-N western blot. Upper and lower arrows indicate positions of GFP–CENP-N and endogenous CENP-N, respectively. Quantification of each band is presented in Supplementary Information, Fig. S4a. (**d**) Co-immunoprecipitation of CENP-H, K and A from micrococcal-nuclease solubilized chromatin with CENP-N mutants. Anti-GFP immunoprecipitates (IP) from each cell line were probed with the indicated antibodies. Seven times more nuclear extract from the CENP-N$^{\Delta C}$ cell line than the wild-type cell line was required to achieve equal levels of CENP-N in the immunoprecipitations. Quantification of each band is presented in Supplementary Information, Fig. S4b. (**e**) CENP-N binds to CENP-L. 6×Myc-CENP-L, L and N were expressed and labelled with ^{35}S-methionine and the indicated proteins were mixed at equal stoichiometry. Of each mixture, 20% was resolved as input (left). The remaining 80% was immunoprecipitated with anti-Myc antibodies (right). Two background bands (asterisks) were present in the input reactions. (**f**) CENP-L binding requires the C terminus of CENP-N. Immunoprecipitations were identical to those in **e**, except that equal amounts of wild-type CENP-N or the indicated CENP-N mutant was used in each reaction.

Depletion of CENP-N with siRNA causes defects in kinetochore assembly and chromosome congression during metaphase, and results in the loss of most other CCAN components from the centromere[5,14]. However, the depletion of other CCAN subunits, including CENP-H, I and K, results in mitotic phenotypes similar to those caused by CENP-N depletion[4,5,14,15]. Furthermore, CENP-N co-purifies with CENP-H, I, M, K, L and T, which are all likely to be interdependent for centromere localization, as indicated by pairwise dependency relationships[4,5,14,15]. Accordingly, a specific function for CENP-N in recognizing centromeric chromatin *in vivo* cannot be inferred from siRNA-based studies.

To directly determine the role of CENP-A nucleosome binding by CENP-N in centromere assembly, we generated CENP-N mutants that had specifically reduced CENP-A-nucleosome binding affinity. Conserved charged and polar amino acids within CENP-N were changed to alanine, based on sequence alignments of CENP-N orthologues from several species (Supplementary Information, Fig. S2a). Following an initial characterization of the mutants *in vitro* and *in vivo* (Supplementary Information, Fig. S3 and Table S1), two of the point mutants, CENP-N^{R11A} and CENP-N^{R196A}, were selected for detailed analysis. Both of these mutants showed reduced CENP-A-nucleosome binding when compared with wild-type CENP-N (Fig. 2a). Dose-response experiments indicated that the CENP-N^{R11A} and CENP-N^{R196A} mutants had a 6-fold and a 2-fold reduction in CENP-A-nucleosome binding, respectively (Fig. 2b; Supplementary Information, Table S1), suggesting that both Arg 11 and Arg 196 in CENP-N contribute to the recognition of CENP-A nucleosomes. In addition, the carboxyl terminus of CENP-N (amino-acids 289–339 in human CENP-N) is more highly conserved among vertebrates than the rest of the protein (Fig. S2b). We therefore constructed a truncation mutant of CENP-N, lacking the C terminus (CENP-N$^{\Delta C}$). The CENP-N$^{\Delta C}$ mutant did not affect nucleosome binding when compared with wild-type CENP-N (Fig. 2a, b).

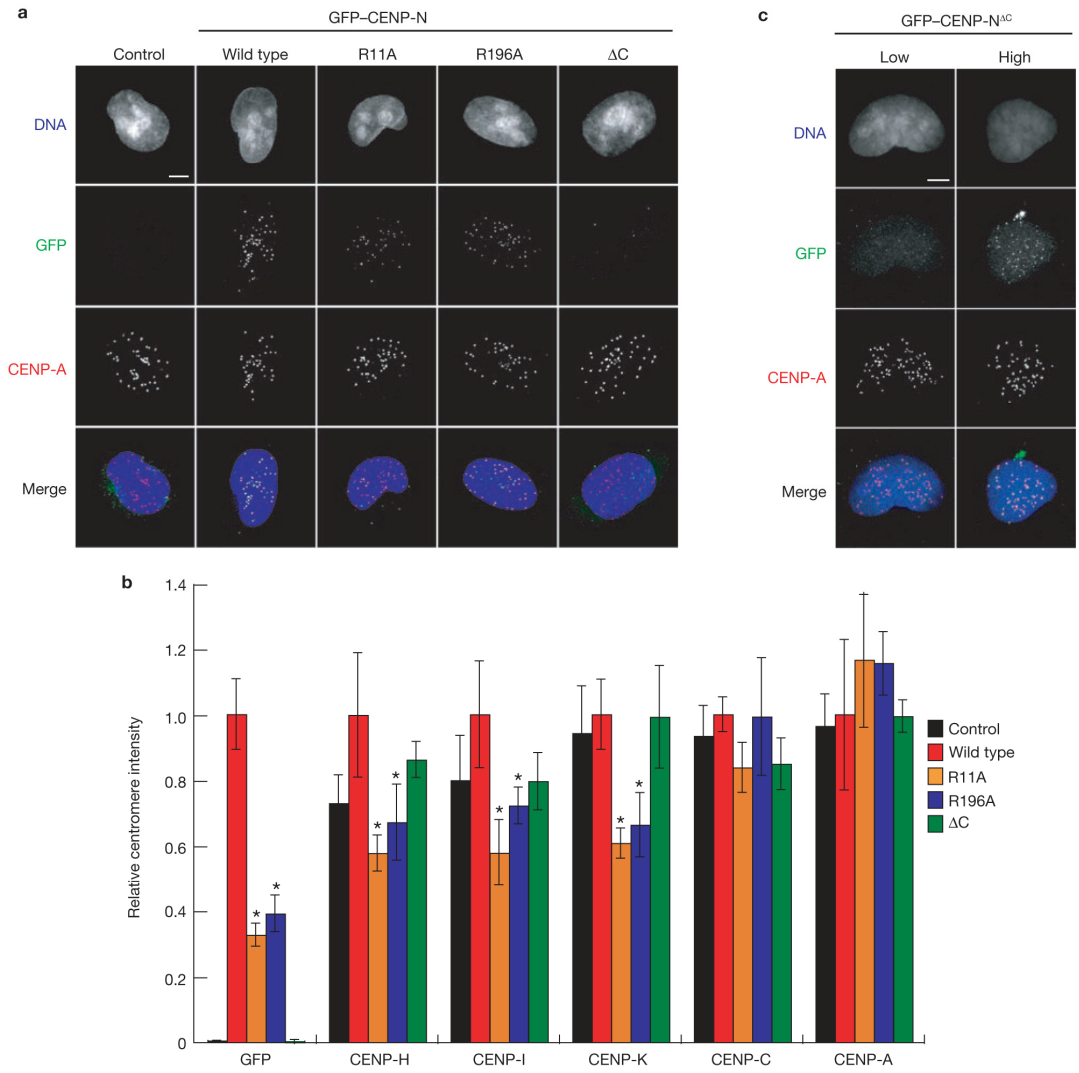

Figure 3 CENP-N mutants show centromere assembly defects. (**a**) Representative images of control HEK293 cells and stable cell lines expressing wild-type or the indicated mutant CENP-N. Scale bar, 5 μm. (**b**) CENP-N mutants cause centromere assembly defects. The fluorescence intensity of the indicated centromere protein at individual centromeres in stable cell lines was measured (see Methods). Data are mean ± s.e.m. from three independent experiments, including > 300 centromeres from > 20 cells for each cell line, $*P < 0.05$ significance compared with wildtype, as determined by Student's *t*-test. (**c**) The C terminus of CENP-N is not required for centromere localization. Representative images from transiently transfected HeLa cells show the localization of GFP–CENP-N$^{\Delta C}$ in low and high expressing cells. Scale bar, 5 μm.

We generated stable HEK293 cell lines that expressed green-fluorescent protein (GFP) fusions to either wild-type CENP-N or the CENP-N mutants to determine how changing the affinity of CENP-N for the CENP-A nucleosome affected CENP-N localization and centromere assembly *in vivo*. Western blotting indicated that each cell line expressed comparable levels of the respective GFP–CENP-N protein, with the exception of the CENP-N$^{\Delta C}$ mutant, which was reduced ~7-fold compared with wild-type GFP–CENP-N (Fig. 2c; Supplementary Information Fig. S4a). HEK293 cell lines express CENP-N from a single genomic locus under the same promoter (see Methods), suggesting that the decreased protein level is a general property of the CENP-N$^{\Delta C}$ mutant. Interestingly, while the levels of CENP-H and CENP-K were not affected in any of the stable cells lines, the expression of wild-type GFP–CENP-N or either of the two GFP–CENP-N point mutants caused a significant reduction in endogenous CENP-N protein (Fig. 2c). Such a reduction has also been described in cells depleted of CENP-H, suggesting that CENP-N is unstable when not associated with other CCAN components[14]. We

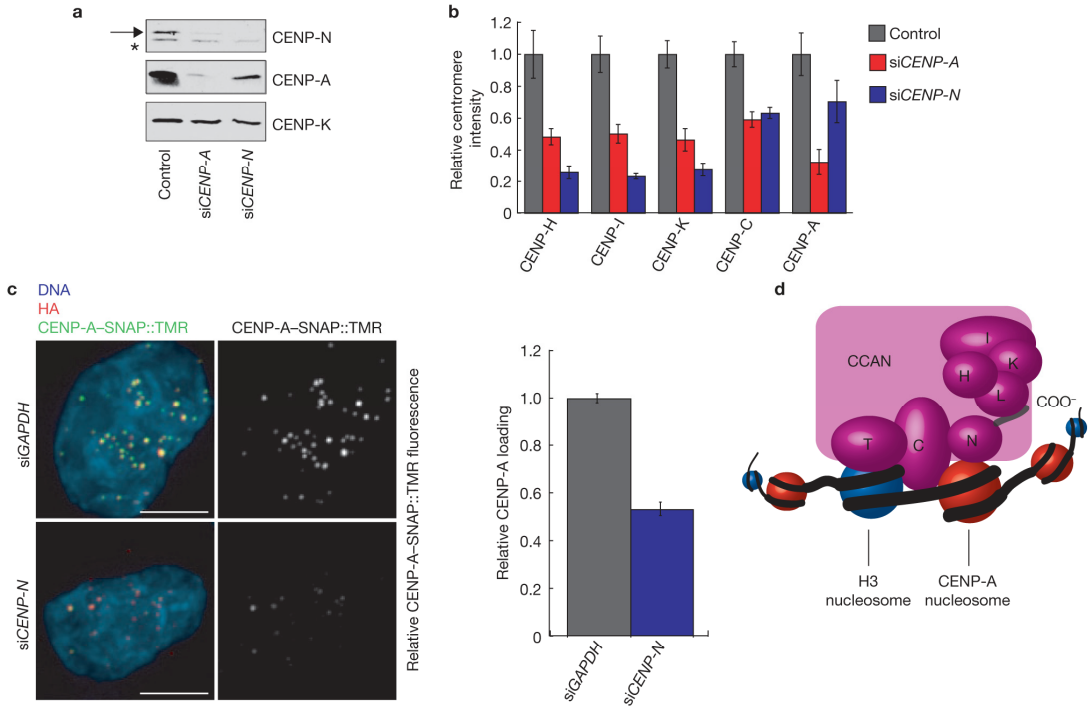

Figure 4 Depletion of CENP-N affects centromere assembly. (**a**) CENP-N depletion leads to a reduction in CENP-A levels. HeLa cells were treated with the indicated siRNAs for 56 h and the abundance of the indicated centromere protein was determined by western blotting. Arrow (top) indicates the position of CENP-N. A background band (asterisk) was present in the anti-CENP-N western blot. CENP-K was included as a loading control. (**b**) CENP-N depletion causes centromere assembly defects. HeLa cells were treated as in **a**, except that the centromere fluorescence intensity of the indicated protein was measured. Data are mean ± s.e.m., from three independent experiments, including > 150 centromeres from > 10 cells for each experiment. (**c**) Reduced CENP-A assembly after *CENP-N* RNAi transfection. CENP-A–SNAP cells were synchronized, transfected with siRNAs against *CENP-N* or *GAPDH* and assayed for CENP-A loading by specifically labelling nascent CENP-A–SNAP using TMR-Star as outlined in Supplementary Information, Fig. S5c. Representative images are shown (left). Haemagglutinin (HA) labels a steady state pool of CENP-A–SNAP and is used as a centromere marker. Scale bar, 5 μm. Data are mean ± s.e.m. from three independent experiments, including the fluorescence intensity of 200 centromeres from 20 cells from each experiment, which was normalized to the *GAPDH* siRNA control. (**d**) A model depicting the multiple roles of CENP-N in CENP-A-nucleosome recognition and centromere assembly, from the recruitment of CCAN proteins to the CENP-N C-terminal region and centromeric chromatin propagation to the CCAN-dependent regulation of CENP-A-nucleosome assembly.

conclude that wild-type GFP–CENP-N and the GFP–CENP-N^{R11A} and GFP–CENP-N^{R196A} mutants effectively replaced endogenous CENP-N in these cell lines.

We directly determined the ability of the CENP-N point mutants to interact with other CCAN proteins and with CENP-A nucleosomes *in vivo*. Immunoprecipitation of wild-type GFP–CENP-N showed that GFP–CENP-N bound to CENP-H, K and A nucleosomes (Fig. 2d; Supplementary Information, Fig. S4b). The GFP–CENP-N^{R11A} and GFP–CENP-N^{R196A} mutants were also associated with CENP-H and CENP-K, although CENP-N^{R196A} bound CENP-H less well than wild-type CENP-N. Importantly, the CENP-N^{R11A} and CENP-N^{R196A} mutants both showed defects in CENP-A-nucleosome binding, the severity of which was consistent with the binding of each mutant to reconstituted CENP-A nucleosomes. Thus, residues Arg 11 and Arg 196 within CENP-N contribute to CENP-A-nucleosome binding *in vitro* and *in vivo*. The comparatively low levels of GFP–CENP-N$^{\Delta C}$ present in stable cells and the inability of this mutant to downregulate endogenous CENP-N protein levels suggests that the GFP–CENP-N$^{\Delta C}$ mutant may not be stably associated with other CCAN proteins. Indeed, GFP–CENP-N$^{\Delta C}$ did not bind to CENP-H, K or A nucleosomes (Fig. 2d).

To understand the mechanism by which the C terminus of CENP-N mediates CENP-N association with CCAN proteins, we expressed epitope-tagged centromere proteins and untagged CENP-N, in reticulocyte extracts, and performed pairwise binding experiments using anti-Myc immunoprecipitation. Myc-tagged CENP-L, but not untagged CENP-L, efficiently co-precipitated with CENP-N, indicating a direct interaction between CENP-N and CENP-L (Fig. 2e). Myc-tagged CENP-L bound the CENP-N^{R11A} and CENP-N^{R196A} mutants as efficiently as it bound wild-type CENP-N (Fig. 2f), consistent with the efficient association of these CENP-N mutants with CENP-H and CENP-K *in vivo*. However, the GFP–CENP-N$^{\Delta C}$ mutant did not bind to CENP-L. These data suggest that CENP-N associates with other CCAN components through a direct interaction with CENP-L that requires the highly conserved C terminus of CENP-N.

We compared the localization of CENP-N^{R11A}, CENP-N^{R196A} and CENP-N$^{\Delta C}$ with wild-type CENP-N. Wild-type GFP–CENP-N localized

exclusively to centromeres as indicated by colocalization with endogenous CENP-A (Fig. 3a). CENP-N^{R11A} and CENP-N^{R196A} also localized to centromeres but did so inefficiently. Quantification showed that the levels of the CENP-N^{R11A} and CENP-N^{R196A} at centromeres was reduced to 32 ± 4% and 39 ± 6%, respectively, from the wild type (Fig. 3b). The difference in localization efficiency between the CENP-N^{R11A} and CENP-N^{R196A} mutants was not as great as would be predicted from their relative CENP-A-nucleosome binding affinities (Fig. 2b, d), suggesting that the other CCAN proteins probably contribute to CENP-N localization efficiency *in vivo*. Nevertheless, these data show that mutations that reduce CENP-A-nucleosome binding by CENP-N result in quantitative defects in the centromere-specific localization of CENP-N.

We did not detect the CENP-N$^{\Delta C}$ mutant at centromeres in our stable cell line, suggesting that association with other CCAN subunits is an important step in the recruitment of CENP-N to centromeres (Fig. 3a, b). However, transiently transfected cells expressing the GFP–CENP-N$^{\Delta C}$ mutant from a strong promoter occasionally contained detectable levels of the mutant protein at centromeres (Fig. 3c). Thus, the C terminus of CENP-N is not absolutely required for CENP-N centromere localization. Instead, our data indicate that association with other CCAN components stabilizes CENP-N and probably increases the efficiency of CENP-N centromere localization.

Mutations in CENP-N that affected CENP-A-nucleosome binding caused dominant defects in centromere assembly in our stable cell lines. Quantification of CENP-H, I and K in cells expressing CENP-N^{R11A} and CENP-N^{R196A} indicated that the levels of each protein at centromeres in interphase cells was significantly reduced when compared with levels in cells expressing wild-type CENP-N (Fig. 3b). Thus, reducing the level of CENP-N at centromeres led to a reduction in the levels of a subset of other CCAN subunits. The defect in CENP-H, I and K localization in the mutant cells was not as severe as that of CENP-N itself, suggesting that a small amount of remaining endogenous CENP-N may also contribute to centromere assembly in these cell lines (Fig. 3b). CENP-N^{R11A} and CENP-N^{R196A} did not alter the levels of CENP-A or CENP-C at centromeres.

Next, we examined the dependence of CENP-H, I and K localization on CENP-N by depleting CENP-N with siRNA in HeLa cells (Fig. 4a; Supplementary Information, Fig. S5a). CENP-N depletion led to a substantial reduction in the levels of CENP-H, I and K at centromeres (Fig. 4b), consistent with previous studies demonstrating CENP-H reduction in cells depleted of CENP-N (ref. 14). The localization defects of CENP-H, I and K in CENP-N-depleted cells were more severe than in cells depleted of CENP-A, indicating that these defects are not an indirect consequence of the reduced CENP-A levels in the CENP-N-depleted cells (Fig. 4b, see below). CENP-C localization to centromeres was also reduced in CENP-N-depleted cells, but not to the same extent as other CCAN proteins (Fig. 4b). Thus, the dependence of CCAN protein localization on CENP-N function was similar between CENP-N-depleted cells and stable cell lines expressing CENP-N mutants with CENP-A-nucleosome binding defects.

Depletion of CENP-N caused a reduction in total levels of CENP-A (Fig. 4a) and CENP-A levels at centromeres (Fig. 4b; Supplementary Information, Fig. S5b). CENP-N is therefore required for the maintenance of centromeric chromatin. Similar defects have been described in *Schizosaccharomyces pombe* cells with mutations in the CENP-N orthologue Mis15, suggesting that CENP-N function is evolutionarily conserved[16]. CENP-H, I and K are required for the deposition of newly synthesized CENP-A at centromeres[4]. We therefore asked whether the reduction in CENP-A levels within centromeric chromatin in CENP-N-depleted cells was due to a failure to load new CENP-A at centromeres. To address this we used the pulse labelling method, based on the SNAP-tag, to determine the fate of newly synthesized protein. Using this strategy, the assembly of nascent CENP-A has been shown previously to be restricted to early G1 phase[17]. The siRNA-mediated reduction of CENP-N levels resulted in a significant reduction in the recruitment of newly synthesized CENP-A–SNAP to centromeres (Fig. 4c; Supplementary Information, Fig. S5c), indicating that the loss of steady-state levels of centromeric CENP-A is caused, at least in part, by a defect in CENP-A assembly.

We have identified CENP-N as the first protein to bind specifically to CENP-A nucleosomes and shown that the direct binding of CENP-A nucleosomes by CENP-N is required for centromere assembly. The DNA sequence-independent binding of CENP-A nucleosomes by CENP-N suggests that CENP-N recognizes the epigenetic mark in chromatin that specifies centromere identity. Importantly, whereas CENP-N targets directly to CENP-A nucleosomes, we found that CENP-N itself is required for recruiting new CENP-A to the centromere, suggesting that CENP-N is part of a feedback loop responsible for propagating centromeric chromatin in dividing cells (Fig. 4d).

CENP-N mutants with a 2-fold or 6-fold reduction in apparent binding affinity for CENP-A nucleosomes, compared with wild-type CENP-N, localized equally efficiently to centromeres *in vivo*, suggesting that CCAN proteins other than CENP-N may also provide direct interactions with chromatin that are important for centromere assembly. The interdependence of several CCAN proteins, including CENP-N and CENP-T, for centromere localization is consistent with this possibility[5]. A complex of CENP-T and CENP-W was recently shown to bind directly to DNA *in vitro* and it associates with histone H3-containing nucleosomes[18]. CENP-N and the CENP-T–W complex are therefore likely to cooperate in providing multiple distinct chromatin contacts that are required for the localization of a subset of other CCAN proteins, including CENP-H, I and K, to centromeres.

CENP-C assembly at centromeres is less sensitive to CENP-N depletion than the other CCAN proteins we examined, consistent with previous results suggesting that CENP-I and CENP-C are independently recruited to centromeric chromatin in human cells[19]. Nevertheless, CENP-A is required for both CENP-N and CENP-C centromere localization, indicating that several centromere localization pathways downstream of CENP-A exist[5,20]. CENP-C has been shown previously to bind directly to DNA *in vitro*, but it is unclear how such an activity could translate into the centromere-specific localization observed for CENP-C *in vivo*[21,22]. Identifying the molecular mechanisms by which CENP-C is recruited to CENP-A chromatin in the absence of CENP-N, and understanding how these distinct centromere-recognition pathways are integrated at the level of chromatin will provide important insights into centromere assembly and structure. □

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturecellbiology/.

Note: Supplementary Information is available on the Nature Cell Biology website.

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AUTHOR CONTRIBUTIONS

C.W.C. and A.F.S. designed the experiments and wrote the manuscript; C.W.C. performed all the experiments except those presented in Figure 4c, which were performed by M.C.C.S. and L.E.T.J.; and K.G. purified histones and helped with nucleosome assembly.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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METHODS

cDNA isolation and expression. cDNAs encoding CENP-C, N, K, T, and I were amplified from HeLa cell mRNA with gene-specific oligonucleotides using the Super-Script III kit (Invitrogen) and cloned by blunt-end ligation into pCR4 Blunt-TOPO using the Zero-Blunt cloning kit (Invitrogen) according to the manufacturers instructions. cDNAs encoding CENP-B, CENP-H, CENP-L, CENP-M, CENP-O, CENP-P, CENP-Q, CENP-R, CENP-S, Mis18α, Mis18β and Mis18BP1/KNL2 were produced by gene synthesis (DNA 2.0, Palo Alto). All cDNAs were sequenced and subcloned into the AscI and PacI sites of a modified pCS2+-based plasmid for coupled transcription and translation in reticulocyte lysates (Promega). ^{34}S-methionine (Amersham) was added to specifically label each protein. The abundance of the centromere proteins was estimated using western blotting.

Histone expression. Histones H2A, H2B, H3 and H4 were expressed, purified and refolded as described previously[23], except that the soluble H3/H4 tetramer and H2A/H2B dimer, rather than the histone octamer, were assembled and frozen on liquid nitrogen. CENP-A was expressed as a soluble tetramer with histone H4 as described previously[11]. Briefly, BL21 bacterial cells (6 l), transformed with a bi-cistronic vector encoding human CENP-A and *Xenopus laevis* histone H4, were grown to optical density (OD) 600 = 0.2 at 37 °C. The culture was then switched to 23 °C and grown until reaching OD600 = 0.6, at which point IPTG (isopropyl-β-D-thiogalactoside) was added to 0.3 mM for 6 h to induce protein expression. The cells were spun down and frozen directly on liquid nitrogen. Cell pellet was resuspended in 100 ml of lysis buffer (10 mM KPO_4, 0.9 M NaCl and 10 mM β-mercaptoethanol — BME — and 1 mM phenylmethylsulphonyl fluoride — PMSF — at pH 6.8), sonicated and centrifuged at 100,000*g* to clarify the extract. The high-speed supernatant was added to a hydroxyapatite column, which was washed with five column volumes of lysis buffer and eluted with a 0.9 M to 3.5 M NaCl gradient in KPO_4 (10 mM at pH 6.8). Soluble CENP-A/H4 typically elutes in a broad peak between 1.5 and 3.5 M NaCl. Fractions containing CENP-A/H4 tetramers were pooled and dialysed two times against SP-loading buffer (10 mM Tris-HCl, 0.75 M NaCl, 10 mM BME and 0.5 mM EDTA at pH 7.4). The dialysate was loaded onto a 1 ml Hi-Trap SP column (Amershan) and eluted with a 0.75 to 2 M NaCl gradient in Tris-HCl (10 mM) and EDTA (0.5 mM at pH 7.4). Fractions containing CENP-A/H4 were pooled, concentrated and added to a Sephadex 200 gel-filtration column equilibrated with Tris-HCl (10 mM), NaCl (2 M) and EDTA (0.5 mM at pH 7.4). Peak fractions were pooled, concentrated and frozen down for nucleosome reconstitution. Approximately 5 mg of CENP-A/H4 tetramer can be expected from 6 l of cells.

Nucleosome reconstitution. DNA (186 bp) containing the centromere repeat or the 601 nucleosome-positioning sequence was generated by polymerase chain reaction (PCR). CENP-A/H4 tetramer, H2A/H2B dimer and DNA were mixed at a stoichiometry of 1.1:2.2:1 in high-salt buffer (10 mM Tris-HCl, 2 M NaCl and 0.5 mM EDTA at pH 7.4) and the salt concentration was slowly lowered to 2.5 mM NaCl over ~60 h by gradient dialysis. Mononucleosomes were then purified on a 5 ml 5-30% glycerol gradient as described previously[23]. Fractions containing pure mononucleosomes were concentrated and stored at 4 °C.

Gel shift assays. Centromere proteins were run out alone or in the presence of the indicated nucleosome on 5% acrylamide gels in 0.5× TBE for 70 min at 10 mA. Typical binding reactions were carried out for 30 min at room temperature and contained the indicated concentrations of nucleosome and 0.5–1 μl of *in vitro* transcription/translation (ivt) mix in Tris-HCl (10 mM) and glycerol (20%) at pH 7.4. Binding reactions were loaded directly on the gel. After electrophoresis, gels were first stained with ethidium bromide or SYBR-gold (Invitrogen) to visualize and/or quantify the nucleosomes, followed by staining with Coomassie blue to visualize proteins. Gels were then dried and the ^{35}S-methionine-labelled centromere proteins were visualized and quantified with a phosphorimager.

CENP-N mutagenesis and stable cell lines. Point mutants in CENP-N were generated with a site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions. Stable cell lines were created by co-transfecting HEK293 flp-in target cells (Invitrogen) with pOG44 and a modified version of pcDNA5/FRT engineered to express CENP-N with an N-terminal GFP-tag according to the manufacturer's instructions. For western blotting and co-immunoprecipitation experiments, nuclei were isolated from ~5×10^7 cells and chromatin was solubilized with micrococcal nuclease as described previously[5,8]. After micrococcal nuclease treatment, extracts were centrifuged at 16,000*g* for 10 min in a microfuge and the concentration of each supernatant was determined using Bradford reagent (Biorad). The supernatant (10 μg) was loaded on a 12.5% poly-acylamide gel to determine the levels of the centromere proteins present (Fig. 2c) and the remaining extract (~2 mg) was used in anti-GFP immunoprecipitations. For CENP-A immunofluorescence, cells were fixed with methanol at -20 °C for 2 min without previous extraction and processed using standard techniques. For staining CCAN proteins, HEK293 cells were pre-extracted with 0.1% Triton X-100 in PBS for 1 min and then fixed for 10 min in PBS + 4% formaldehyde and processed using standard techniques. Similar methods were used for staining CCAN proteins in HeLa cells except that cells were pre-extracted for 3 min in 0.3% Triton X-100 before fixation.

siRNA. *CENP-N* (5'-GUAAAUUUCCGACAGAGAAUU-3', 5'-CUACCUACGUGGUGUACUAUU-3', 5'-GAUAUUACCGAAAUGAAGAUU-3', 5'-CCAGAAAGUUUGGGAUGUUUU-3') and *CENP-A* (5'-AACACAGUCGGCGGAGACAAG-3') siRNAs (Dharmacon) were used according to the manufacturer's instructions. Buffer alone or *GAPDH* siRNA was used in control experiments. Cells were processed for immunofluorescence as described above. For western blots, cell extracts were prepared by resuspending cells (20 mM HEPES, 0.5 M NaCl, 1% Triton X-100, 1 mM dithiothreitol, 10 mM EDTA, 10 mM EGTA and 1 mM PMSF), followed by centrifugation for 10 min at 16,000 *g* in a microfuge. Protein concentration in each extract was determined using Bradford reagent (Biorad) and identical amounts of total protein were loaded in each well of the gel.

SNAP pulse labelling. HeLa cells stably expressing near endogenous levels of CENP-A-SNAP-3×HA[17] were treated with thymidine (2 mM) for 17 h to arrest cells in S phase. Following release in deoxycytidine (24 μM) for 3 h, cells were transfected with CENP-N and GAPDH siRNA pools (Dharmacon) according to the manufacturer's instructions. At 9 h after release from thymidine, fresh thymidine was added to synchronize cells at the next G1/S boundary. SNAP-tag was quenched with non-fluorescent BTP (Covalys) after which cells were released into S phase. Newly synthesized CENP-A-SNAP was labelled 7.5 h after release (in G2 phase) with TMR-Star (Covalys), cells were allowed to proceed through the cell cycle for CENP-A assembly and were collected at the next G1/S boundary by the addition of thymidine. Cells were fixed (without pre extraction) and processed for microscopy (see Supplementary Information, Fig. S5c for schematic).

Microscopy. Stacks of fixed cell immunofluorescence images encompassing the entire cell (or cells) were captured at 0.2 μm axial steps using a motorized stage mounted on an Olympus IX70 microsope. Immunofluorescent images were acquired using a ×60 1.4NA PlanApo objective and a CoolSnap-HQ CCD camera (Photometrics) on a DeltaVision Spectris system (Applied Precision). TMR-Star-labelled CENP-A-SNAP images were acquired using a ×100 1.4 NA UPlanSApo objective and a Cascade2 EMCCD camera (Photometrics). Centromere fluorescence intensity in each channel was quantified as described except that intensity measurements were performed on non-deconvolved maximal intensity projections of each *z*-series[24]. The images presented are maximum intensity projections of deconvolved images.

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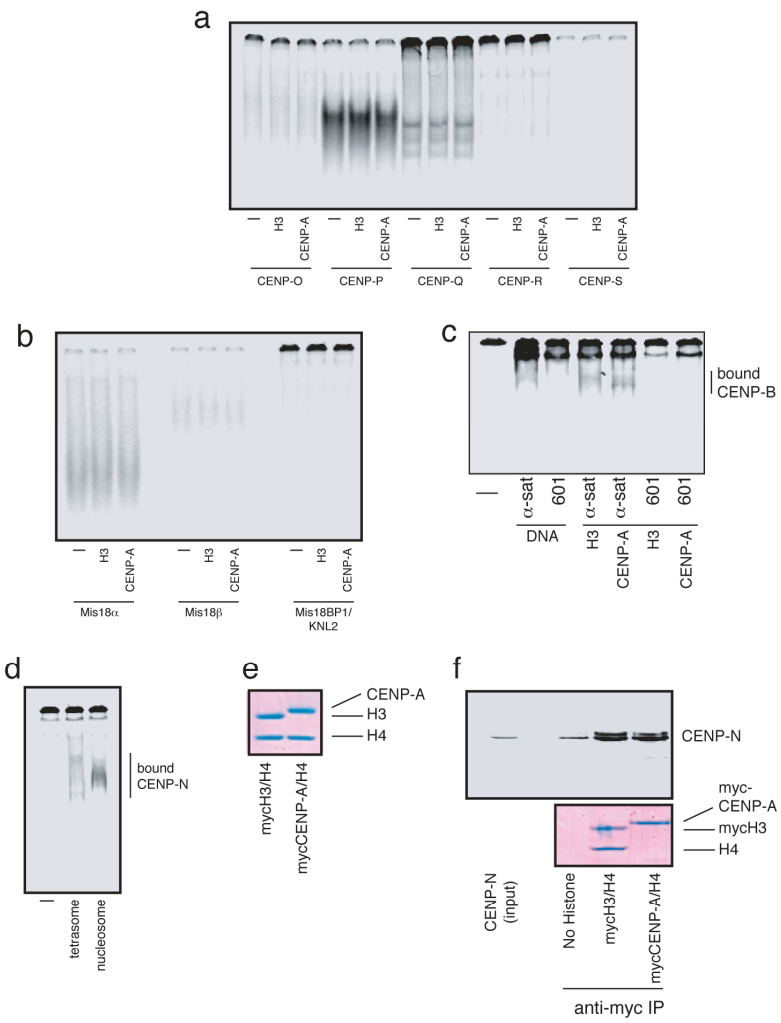

Figure S1 CENP-A nucleosome binding assay. (**a**,**b**) CENP-A or H3 nucleosomes or buffer alone (-) were incubated with the indicated centromere proteins and resolved on native gels as described in Figure 1c and 1d. (**c**) An experiment identical to that presented in Figure 2a was performed except that the nucleosome-binding requirement of CENP-B (1 nM) was determined. (**d**) An experiment identical to that in Figure 1a was performed with ^{35}S-labeled CENP-N (~10 nM) except that 100nM CENP-A/H4 tetrasomes (containing CENP-A/H4 and α-satellite DNA) or 100 nM CENP-A nucleosomes were used. The control reaction (-) contained buffer only. (**e**) Purified myc-CENP-A/H4 and myc-H3/H4 tetramers used in anti-myc immunoprecipitations were resolved on a 17.5% gel and stained with coomassie blue. (**f**) Anti-myc immunoprecipitation of purified myc-CENP-A/H4 and myc-H3/H4 tetramers with ^{35}S-labeled CENP-N. Input represents 10% of total CENP-N in each immunoprecipitation. The myc-CENP-A/H4 tetramer (bottom panel) dissociates at the low salt concentrations used in these experiments.

a

```
                                                  *   *
HsCENP-N  ------------------MDETVAEFIKRTILKIPMNELTTILKAWDFLSENQLQTVNFR
BtCENP-N  ------------------MDETLAEFFRRTILKIPMTEMMTILKTWNFMSENQLQTVNFR
MmCENP-N  ------------------MKENVAEFLRRTILKIPLSEMKSILEAWDFLSEDQLQTINLK
GgCENP-N  ------------------MDEVIVEYIRRTVLKIPRDEIMAVLQKWGFLSEAQLQTINFR
XlCENP-N  MAPAAEIVQKRKRQSALVMDEWLAEFIKRTILKLPFSETATILKTWGFLTESELQTFTLR
             *                    *
HsCENP-N  QRKESVVQHLIHLCEEKRASISDAALLDIIYMQFHQHQKVWDVFQMSKGPGEDVDLFDMK
BtCENP-N  QRKESIVQDLVLLCEENHASLNDAAHLDIIYTQFHRHQKIWDVFQMSKAPGDDIDLFDME
MmCENP-N  QRKDYLAQEVILLCEDKRASLDDVVLLDIVYTQFHRHQKLWNVFQMSKEPGEDVDLFDME
GgCENP-N  QTKEGISHSVAQLCEESSADLKQAALLDIIYNHIYPNKRVWSVYHMNKT-GEETDFFDFR
XlCENP-N  YPKEVTATEVVRFCEARNATLDHAAALDLVFNHAYSNKKTWTVYQMSKRLESENDLFDAS
                                *                   *
HsCENP-N  QFKNSFKKILQRALKNVTVSFRETEENAVWIRIAWGTQYTKPNQYKPTYVVYYSQTPYAF
BtCENP-N  QFKSSFKKILQRALKNVTVSFRDAEENSVWIRIAWGTQYKKPNQYKPAYVVYYSQTPYAF
MmCENP-N  QFQSSFKRILQRALKNVTVSFRVYEKDSVWIRVAWGTQYSQPNQYKPTFVVYYPQTPYAF
GgCENP-N  DFKKKFRRQIQSALINVTINFREYEDNAIWIRIAWGTPYTKPNQYKTSYVVYHSQTPYVF
XlCENP-N  EFKLQFKKSIHAVSKNVTINFKEFG-EALWIRIAWGTHNSRPNQYKATFAVYHSQTPYVF
                                                *
HsCENP-N  TSSSMLRRNTPLLGQALTIASKHHQIVKMDLRSRYLDSLKAIVFKQYNQTFETHNSTTPL
BtCENP-N  TSSSRLKSNLPLLGQALTVASKHHQIVKMDLRSRYLDSLKAIVFKQYNQSFETHNCTTSL
MmCENP-N  ISSCHLKNTVPLLHQALKVASKHHQIVHLDLRSRHLDSLKAIVFREYNQTCENYSSTTSL
GgCENP-N  ISASVLRSNLPLLCQAMVVASNYHDIHEMELRSHCLNSLKDIVFKRYSQNFQTN---YPL
XlCENP-N  ITG-LGKACQPLMCQALVIASKYSQIQEMELKSRCLESLKDIVFKRFNQPFSSHHSKPHE

HsCENP-N  QERSLGLDINMDSRIIHENIVEKERVQRITQETFGDYPQPQLEFAQYKLETKFKSG-LNG
BtCENP-N  QEGSLGLDINMDSRIIHENKVEKERVQRVTQEIFGDYPQPRLEFAQYKLETKFKSD-LNG
MmCENP-N  QEASL--SMCLDSKITHENTEEKVRVHRVTQETFGTYPQPQLEFAQYKLETKFKSN-IGG
GgCENP-N  QERNV-LTENVDLRINDENRSEKERIYRLNQESFGNGPQPKLDFAQYKLETMFKSD-PKW
XlCENP-N  KALTP---NIVDPRVTYENMREKDRVYHLTCETFGEGPLPKLELASYKLETMFKADSAMS

HsCENP-N  SILAERKEPLRCLIKFSSPHLLEALKSLAPAGIADAPLSPLLTCIPNKRMNYFKIRDK--
BtCENP-N  GILAEREEPLRCLVKFSSPHLLEALKSLAPAGIADAPLSPLLTCIPNKGKNYFKIRDK--
MmCENP-N  GLLADRKEPFRCLVKFSSPHLLEALKSLAPAGIADAPLSPLLTCIPSKKMNYFKIRDK--
GgCENP-N  DVLE-KKEPFRCLVKFSSPHLLESLKSLAPAGLADAPLSPLLTCIPQKARNYFKIREKKS
XlCENP-N  GNLTAVNEPFRCVVKFSSPHLLEAIRSLAPAGIAEAPISTLLSCIPHKARNSFKITEKRS

HsCENP-N  -----------
BtCENP-N  -----------
MmCENP-N  -----------
GgCENP-N  LHPGSFVSP--
XlCENP-N  MHPSSSQPTNV
```

b

	% Identity to HsCENP-N	
	1-289	290-339
BtCENP-N	84	93
MmCENP-N	70	93
GgCENP-N	56	83
XlCENP-N	70	68

Figure S2 Alignment of CENP-N orthologs. (**a**) CENP-N orthologs from several vertebrate species were aligned using Clustal W. Residues identical to those in human CENP-N are shaded in grey. Residues that were mutated to alanine in human CENP-N are indicated (*). The residues deleted in the CENP-NΔC truncation mutant are outlined in blue. (**b**) Comparison of the percent identity between human CENP-N and other vertebrate CENP-N orthologs over amino acids 1-289 or 290-339 of human CENP-N.

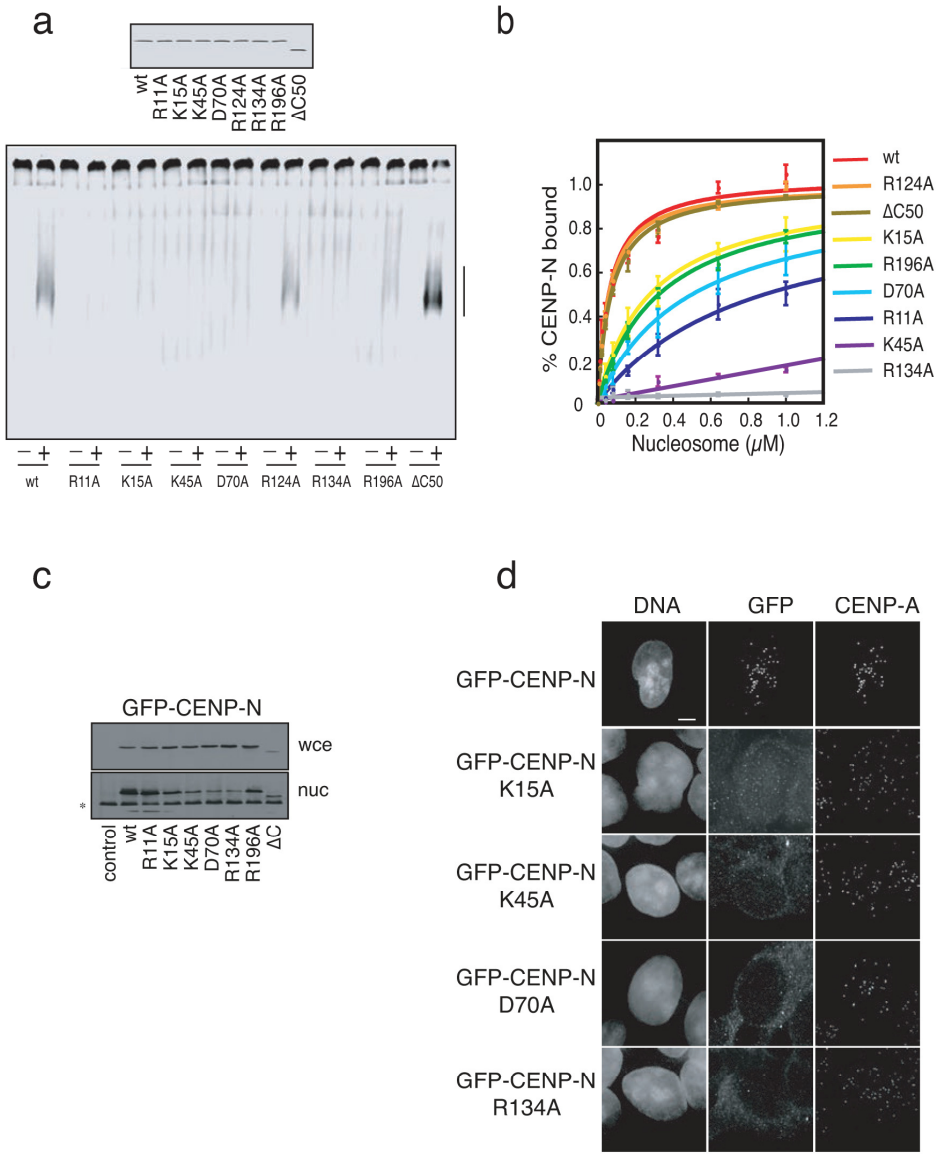

Figure S3 Initial characterization CENP-N point mutants. (**a**) CENP-N mutants exhibit a range of affinities for CENP-A nucleosomes. CENP-N (wt) or individual CENP-N mutants were expressed in reticulocyte extract and resolved by SDS-PAGE (top panel). Each mutant was assayed for its ability to bind CENP-A nucleosomes (bottom panel). Nucleosome gel shift assays included wildtype CENP-N and each CENP-N mutant alone (-) or in the presence of 300 nM CENP-A nucleosome (+). (**b**) Dose-response experiments for each CENP-N mutant were performed as in A (bottom panel) with increasing concentrations of CENP-A nucleosome added to each reaction (N=3, error bars represent SEM). Data for wildtype CENP-N, and the R11A, R196A and ΔC mutants is reproduced from Figure 2b for comparison. (**c**) Expression of CENP-N mutants in HEK293 cells. Whole-cell extracts (wce) or nuclear extracts (nuc) from stable HEK293 cell lines expressing GFP-CENP-N (wt) or the indicated GFP-CENP-N mutant were separated by SDS-PAGE and western blotted with anti-GFP antibodies. A nonspecific band (*) that migrates faster than GFP-CENP-N is present in the nuclear extracts. (**d**) Representative images of HEK293 stable cells expressing GFP-CENP-N or the indicated GFP-CENP-N mutant. The image of cells expressing GFP-CENP-N is reproduced from Figure 3a and is included for reference.

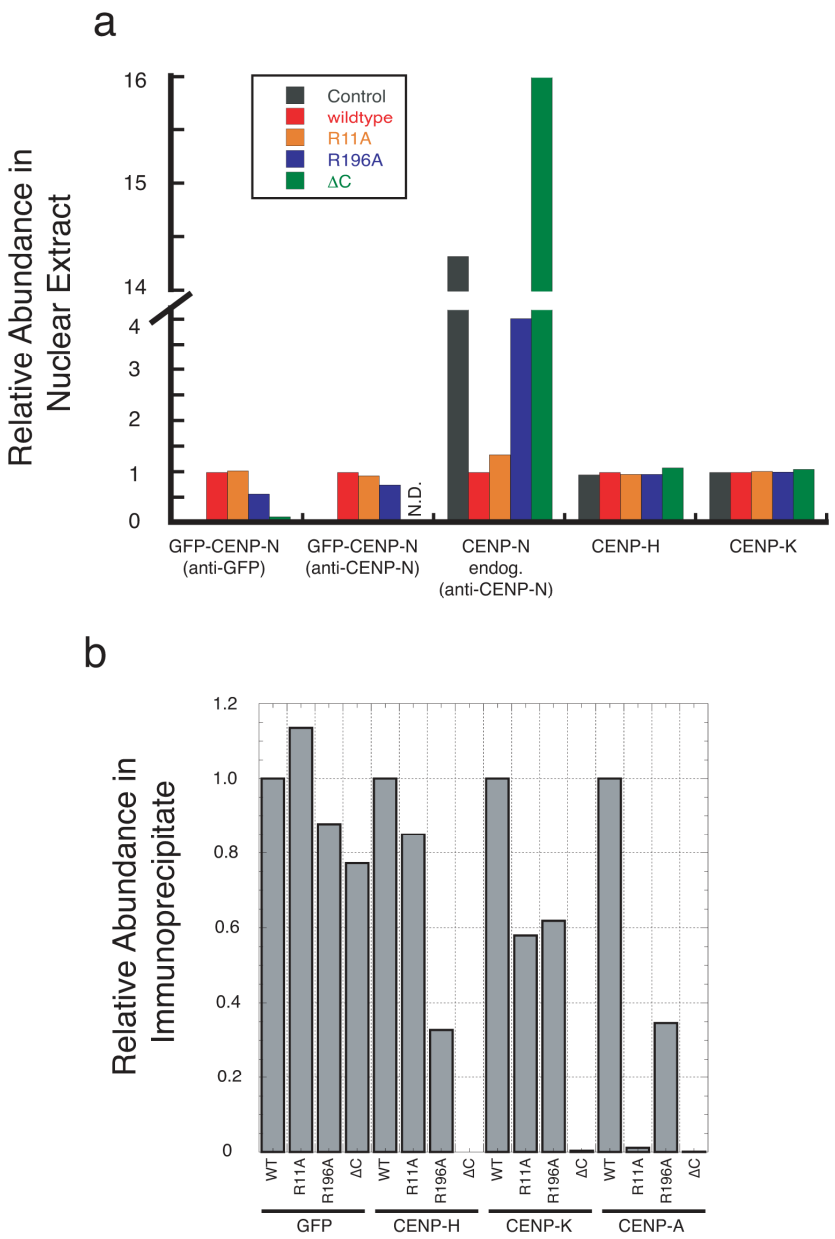

Figure S4 CENP-N mutants show changes in CENP-A nucleosome interaction and CCAN protein association. (**a**) Quantification of the levels GFP-CENP-N, endogenous CENP-N, CENP-H and CENP-K proteins from the western blots shown in Figure 2c. The amount of protein in each case is normalized to the amount of protein presiend in the GFP-CENP-N lane. (**b**) Quantification of GFP-CENP-N wildtype and mutant immunoprecipitations from western blot in Figure 2d. The amount of protein in each case is normalized to the amount present in the GFP-CENP-N wildtype immunoprecipitation.

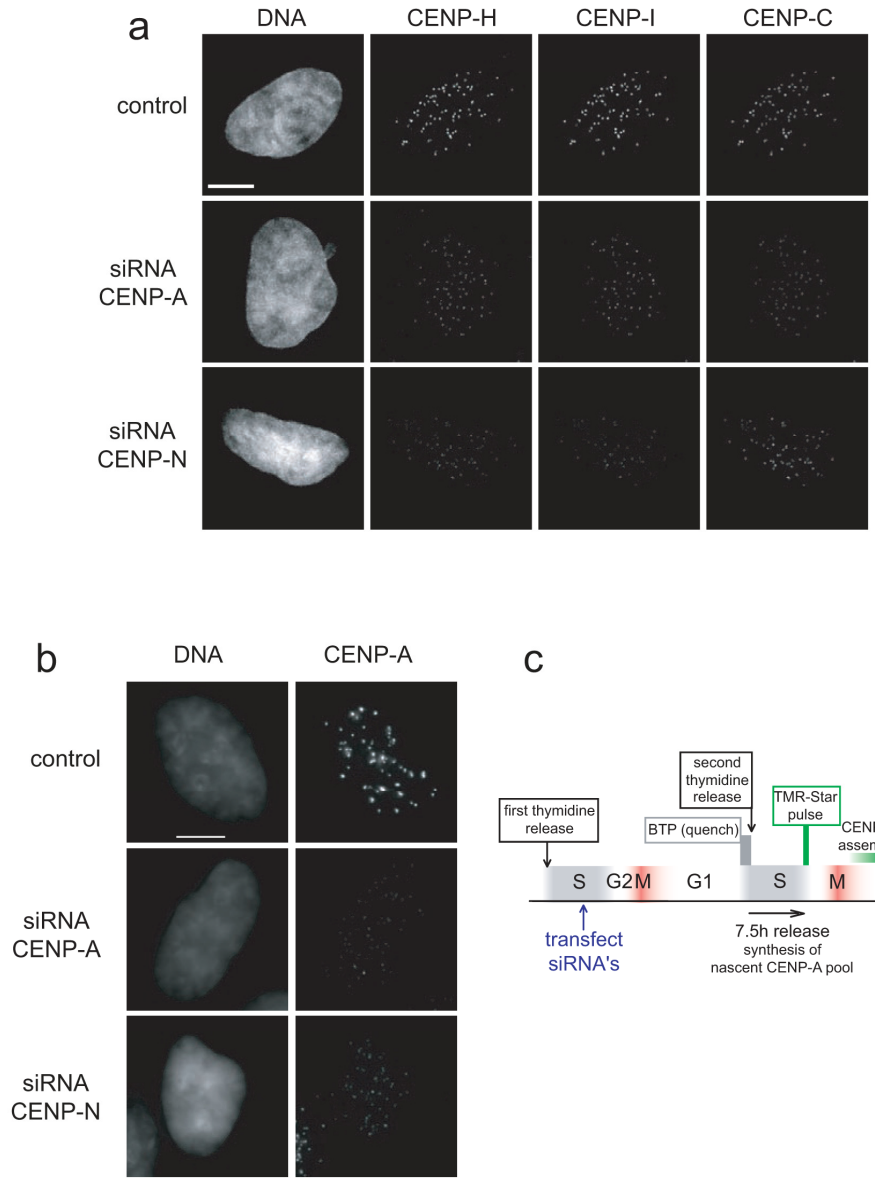

Figure S5 CENP-N is required for centromere assembly. (**a**,**b**) Representative images of control cells or cells depleted of CENP-A or CENP-N with siRNA stained with the indicated antibodies to visualize centromere proteins. Scale bar is 5 μm. (**c**) Experimental scheme for SNAP based CENP-A assembly assay depicting order of synchronization, transfection and SNAP labeling steps described in detail in Material and Methods.

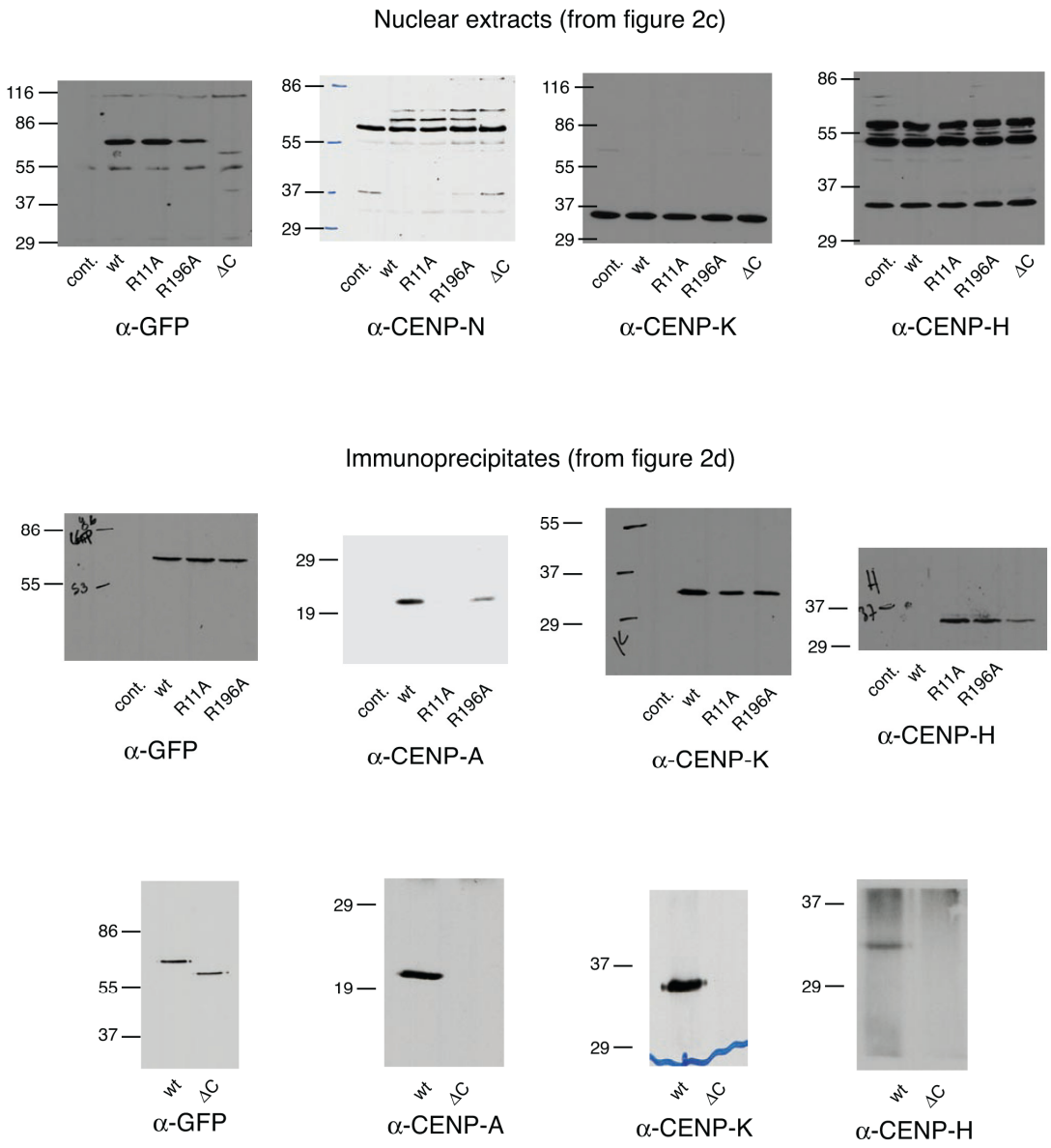

Figure S6 Full scans of western blot images.

CENP-N	K_d apparent (nM)
wt	169±70
R11A	962±259
K15A	297±77
K45A	ND
D70A	546±152
R124A	72±10
R134A	ND
R196A	331±36
ΔC	62±8

Table S1 The apparent dissociation constant of wild-type CENP-N and each CENP-N mutant for reconstituted CENP-A nucleosomes as determined by the dose-response experiments presented in Figure 2b and S5b. Affinites for R134A and K45A were too low to accurately determine a dissociation constant (ND).

Appendix 3 – At the right place at the right time: novel CENP-A binding proteins shed light on centromere assembly

Chromosoma
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MINI-REVIEW

At the right place at the right time: novel CENP-A binding proteins shed light on centromere assembly

Mariana C. C. Silva · Lars E. T. Jansen

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Abstract Centromeres, the chromosomal loci that form the sites of attachment for spindle microtubules during mitosis, are identified by a unique chromatin structure generated by nucleosomes containing the histone H3 variant CENP-A. The apparent epigenetic mode of centromere inheritance across mitotic and meiotic divisions has generated much interest in how CENP-A assembly occurs and how structurally divergent centromeric nucleosomes can specify the centromere complex. Although a substantial number of proteins have been implicated in centromere assembly, factors that can bind CENP-A specifically and deliver nascent protein to the centromere were, thus far, lacking. Several recent reports on experiments in fission yeast and human cells have now shown significant progress on this problem. Here, we discuss these new developments and their implications for epigenetic centromere inheritance.

Introduction

The centromere is a specialized chromosomal locus that drives the assembly of the kinetochore, the structure to which spindle microtubules attach during mitosis and meiosis, allowing accurate chromosome segregation (Allshire and Karpen 2008; Cleveland et al. 2003). Although centromeres are directly associated with chromosomal DNA, specific DNA sequences are neither required nor sufficient for centromere identity in most eukaryotes with the exception of budding yeast (Cheeseman et al. 2002). Rather, centromeres appear to be inherited through an epigenetic mechanism. Key evidence in support of an epigenetically maintained centromere is the generation of neocentromeres in human patients and experimentally in fission yeast on non-centromeric DNA (Ishii et al. 2008; Warburton 2004). Once formed, neocentromeres are mitotically stable and are, in some cases, inherited across generations (Amor et al. 2004). Despite the divergence in size and sequence of centromeric DNA among eukaryotes, the nature of centromeric chromatin is similar across species. All eukaryotes, thus far, feature a unique histone H3 variant, identified in humans as CENP-A (Cse4 in *Saccharomyces cerevisiae*, Cnp1 in *Schizosaccharomyces pombe*, HCP-3 in *Caenorhabditis elegans*, CenH3 in plants and CenH3 or CID in *Drosophila*) that replaces canonical histone H3 in nucleosomes at active centromeres and is responsible for nucleating the centromere/kinetochore complex (Foltz et al. 2006; Liu et al. 2006; Palmer et al. 1987; Regnier et al. 2005; Yoda et al. 2000). Overexpression of CENP-A^{CID} in *Drosophila* leads to misincorporation outside the centromere locus which in turn triggers ectopic recruitment of centromere and kinetochore components supporting the idea that CENP-A is the primary candidate for generating an epigenetic mark that specifies centromere identity and function (Heun et al. 2006).

Amide proton exchange studies have shown CENP-A nucleosomes to be more rigid compared to their canonical counterpart suggesting that, indeed, centromeric chromatin is structurally divergent from the rest of the chromosome (Black et al. 2004, 2007a). Strikingly, structural rigidity can be recapitulated in canonical histone H3 when substituting the 22 amino acids specific to CENP-A within the 40 amino acids that comprise loop1 and α2 helix within its histone

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fold. This domain was termed the CENP-A centromere targeting domain (CATD) as it is sufficient to target histone H3 to the centromere and functionally replace CENP-A (Black et al. 2004, 2007b). Interestingly, the biophysical measurements mentioned above showed, by deuterium exchange, that this domain is locked inside the nucleosome suggesting that CENP-A function is derived from the structural changes it induces in the nucleosome.

If, indeed, the structurally divergent centromeric nucleosome identifies the centromere, two questions become critical. First, epigenetic memory of centromere inheritance across mitotic and perhaps even meiotic divisions implies a positive feedback loop where the assembly of the centromeric nucleosome into chromatin is dependent on nucleosomes inherited from previous divisions. The question of how CENP-A is assembled and maintained is, therefore, a relevant one. Secondly, if the CATD domain of CENP-A is essential yet buried inside the nucleosome, how then is centromeric chromatin recognized by the centromere complex that is assembled on it? Now, reports on experiments in fission yeast and human cells discussed below shed light on both questions.

Structural centromere components affecting CENP-A assembly

The core centromere is comprised of proteins that are associated with CENP-A chromatin throughout the cell cycle referred to as the constitutive centromere-associated network (CCAN) that so far include, in addition to centromeric nucleosomes, 15 proteins; CENP-C, CENP-H, CENP-I, CENP-K through CENP-U, and CENP-W (Cheeseman and Desai 2008; Hori et al. 2008). Three different subsets of this collection of proteins are known as the CENP-A Nucleosome Associated Complex (CENP-A^{NAC}; Foltz et al. 2006), the CENP-H–I complex (Okada et al. 2006; Table 1), and the Interphase Centromere Complex (Izuta et al. 2006). Several members of the CCAN have been shown to affect CENP-A levels at the centromere that include CENP-H, CENP-I, CENP-K, and CENP-M (Okada et al. 2006). In flies, another member of this complex, CENP-C is also required for maintaining CENP-A levels at the centromere, although this requirement appears specific for this species (Erhardt et al. 2008). Members of the human Mis12 complex, although not constitutively centromeric, also influence CENP-A centromere occupancy (Kline et al. 2006).

Now, in a recent issue of Nature Cell Biology, Carroll and co-workers reported that among eight members of the previously described CENP-A nucleosome associated complex (Foltz et al. 2006), only CENP-N directly binds CENP-A nucleosomes in vitro (Carroll et al. 2009). Strikingly, efficient binding occurs only when CENP-A or histone H3 carrying the CATD domain of CENP-A is assembled into nucleosomes. No specific binding is found to H3 nucleosomes or prenucleosomal CENP-A/H4 tetramers. Moreover, CENP-N binding to CENP-A nucleosomes is not dictated by any specific nucleosomal DNA sequence. This leads to the proposal that CENP-N directly recognizes a structural aspect specific to the CENP-A nucleosome induced by the CATD domain that constitutes the epigenetic centromere mark (Fig. 2a). CENP-N is bound in turn by CENP-L, leading the way to the formation of the rest of the centromere complex. Mutations that affect CENP-N binding to the CENP-A nucleosome while retaining the ability to bind CENP-L result in loss of centromere function indicating that CENP-A nucleosome binding is essential for CENP-N function. Like for other members of the CCAN mentioned above, depletion of CENP-N affects CENP-A levels at the centromere. This defect results from an inability to assemble or stabilize newly synthesized CENP-A as measured by SNAP-based pulse labeling experiments (Carroll et al. 2009). It is, at present, unclear how these apparent structural components affect CENP-A assembly. The fact that these proteins are found associated with CENP-A nucleosomes (either directly or indirectly) but not found in soluble fractions of CENP-A (Dunleavy et al. 2009; Foltz et al. 2009) indicates that they do not represent assembly factors per se. Instead, they may serve as a platform for specific CENP-A loading factors to target to centromeres or, alternatively, their complex formation onto centromeric chromatin may stabilize CENP-A nucleosomes. Importantly, all of these proteins are themselves dependent on CENP-A for their localization to the centromere (Carroll et al. 2009; Foltz et al. 2006; Liu et al. 2006). The intriguing implication of this is that one or more members of the CCAN may form the molecular basis for an epigenetic feedback loop to control the propagation of active centromeres (Fig. 2c).

The role of histone chaperones

If not structural centromere components, what then are the activities that escort nascent CENP-A to the centromere? The fission yeast Mis16 protein, homologous to human RbAp46 and RbAp48, is required for CENP-A localization at the centromere (Hayashi et al. 2004). Indeed, both human proteins also affect CENP-A assembly (Dunleavy et al. 2009; Hayashi et al. 2004). Both are members of the CAF-1 complex that is responsible for the assembly of canonical histone H3.1 during S phase (Verreault et al. 1996). RbAp48 is also a member of the HIRA complex, controlling assembly of the H3.3 variant that exchanges into chromatin preferentially at transcriptionally active regions (Tagami et al. 2004). CENP-A, when overexpressed, can interact with RbAp48 in *Drosophila* and human cells (Dunleavy et al. 2009;

Furuyama et al. 2006). However, the fact that RbAp48 is involved in the assembly process of all histone H3 variants suggests that it serves as a general histone chaperone possibly through direct binding to histone H4 that is common to all H3 prenucleosomal complexes. These findings have led to proposals that perhaps the CENP-A assembly process occurs through a default pathway. Centromere specificity is then achieved either by targeting competing histone H3.1 and H3.3 elsewhere (Furuyama et al. 2006) or by generating available sites for CENP-A assembly (e.g., by evicting histone H3 nucleosomes) only at the centromere (Dalal 2009). While these are rather unlikely models, they obviate the need for a specific CENP-A assembly factor. In fission yeast, the Sim3 protein not only binds CENP-A^{Cnp1} but also displays affinity for canonical H3, like RbAp48/46 as well as the Sim3-related human NASP (Dunleavy et al. 2007; Tagami et al. 2004). Although Sim3 appears to prefer CENP-A^{Cnp1} as a partner, it is not centromere-localized and, therefore, has been proposed to act as an intermediate histone chaperone that delivers CENP-A to assembly factors (Dunleavy et al. 2007).

Cell cycle control of CENP-A assembly

A critical advance in determining the mechanism of CENP-A assembly has been the identification of a discrete cell cycle window during which CENP-A loading into chromatin occurs. GFP-CENP-A^{CID} photo-bleaching experiments in rapidly cycling *Drosophila* syncytial embryos showed fluorescence recovery only during a brief window following mitotic exit concurrent with an increase of overall GFP-CENP-A^{CID} levels at the centromere (Schuh et al. 2007). This suggests that centromeric chromatin is assembled during this time window. Direct evidence for the timing of CENP-A assembly came from fluorescent pulse labeling experiments based on SNAP-tagging in human cells that allow for specific visualization of a newly synthesized pool of CENP-A (Jansen et al. 2007). These experiments showed that CENP-A can be expressed at any stage during the cell cycle but will target to centromeres only following exit from mitosis during the first hours of G1 phase (Figs. 1 and 2). This finding was confirmed by photo-bleaching experiments and is consistent with the results in fly embryos (Hemmerich et al. 2008; Schuh et al. 2007). Early G1 loading of new CENP-A, thus, appears to be a general phenomenon across animals. Strikingly, once assembled, CENP-A nucleosomes are extremely stable and are quantitatively recycled during subsequent rounds of DNA replication consistent with a role in heritably maintaining centromere identity (Jansen et al. 2007). Fission yeast cells enter S phase almost immediately following exit from mitosis. It is at this time that CENP-A^{Cnp1} is expressed and assembled suggesting a temporal control similar to that found in higher eukaryotes. However, unlike in fly embryos and human tissue culture cells, there is a second wave of assembly during the fission yeast G2 phase (Takahashi et al. 2005).

Being at the right place at the right time

Given the conserved cell cycle control of CENP-A assembly, one would expect that if specific factors exist that propagate centromeric chromatin, they would be centromere-localized within the appropriate cell cycle window. Indeed, a set of proteins has recently been identified that meet this requirement. One of the founding members of this group, Mis18, was identified in fission yeast and was shown to be required for CENP-A^{Cnp1} localization to the centromere (Hayashi et al. 2004). Strikingly, Mis18 is absent from centromeres during mitosis but accumulates there following mitotic exit. This temporal localization pattern is conserved in a complex of human proteins that include the Mis18 homologs hMis18α and hMis18β as well as an associated protein, M18BP1 that was independently identified as the human homolog of the *C. elegans* KNL-2 protein (Fujita et al. 2007; Maddox et al. 2007). All three proteins arrive at the centromere during anaphase and leave 2–3 h later in mid-G1 (Fujita et al. 2007). Depletion of any of these proteins severely impacts CENP-A localization at the centromere. Importantly, CENP-A assembly closely follows hMis18/HsKNL2 targeting in anaphase ensuing shortly thereafter in telophase (Fig. 1; Fujita et al. 2007; Jansen et al. 2007; Maddox et al. 2007). Despite their suggestive localization pattern and clear role in CENP-A assembly, none of the yeast or human Mis18/HsKNL2 proteins appear to bind to CENP-A directly (Fujita et al. 2007; Hayashi et al. 2004). In addition, they have not been found in proteomic screens for CENP-A nucleosome or prenucleosome binding factors (Dunleavy et al. 2009; Foltz et al. 2006, 2009).

Recently, Rsf-1, a component of the ATP-dependent chromatin remodeling and spacing factor RSF, was added to the list of proteins temporally transiting the centromere. Rsf-1 localization occurs in mid-G1, several hours after the hMis18/HsKNL2 proteins arrive there (Perpelescu et al. 2009). Unlike the (h)Mis18/HsKNL2 proteins, Rsf-1 as well as its partner SNF2h associates with CENP-A chromatin at the mononucleosome level. RSF does not appear to be involved in targeting CENP-A as Rsf-1 localization occurs well after nascent CENP-A arrives at the centromere and Rsf-1 depleted cells show normal CENP-A levels at centromeres. However, centromeric CENP-A is sensitive to salt extraction in these cells. This has led Perpelescu and colleagues to suggest a model in which CENP-A is initially targeted to centromeres in early G1 after which it is assembled into

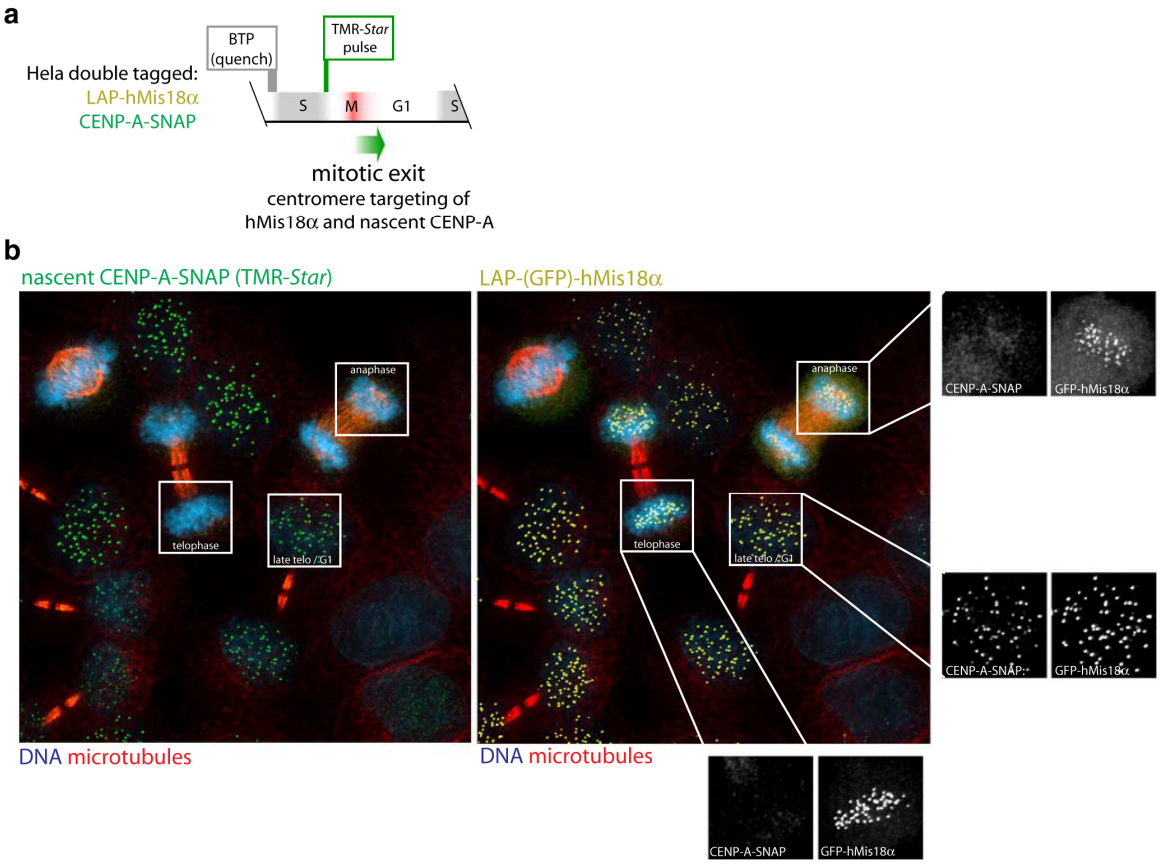

Fig. 1 hMis18α targets to centromeres prior to CENP-A assembly. **a** An S-phase synthesized pool of CENP-A-SNAP was specifically labeled with fluorescent TMR-Star by quench-chase-pulse labeling as described (Jansen et al. 2007) in HeLa cells stably expressing both CENP-A-SNAP and LAP-(GFP)-hMis18α. **b** A single four channel field is shown as two separate images with TMR-labeled nascent CENP-A-SNAP in green and LAP-tagged hMis18α in yellow. *Grayscale blowups* show differential timing of centromere loading (hMis18α in anaphase, newly synthesized CENP-A in telophase). Microtubules and DNA are shown in *red* and *blue*, respectively

chromatin or otherwise stabilized through RSF action. It remains to be demonstrated, however, whether the salt-sensitive pool observed represents newly targeted CENP-A. Nevertheless, consistent with the model proposed, RSF was found to have general nucleosome assembly and/or spacing activity in vitro (LeRoy et al. 1998; Perpelescu et al. 2009). Interestingly, a similar ATP-dependent chromatin remodeling factor, Hrp1 in fission yeast has previously been shown to associate with centromeric repeats and affect centromeric CENP-A^{Cnp1} levels (Walfridsson et al. 2005).

Novel factors binding soluble CENP-A and their function in centromere assembly

If targeting of CENP-A to the centromeres is mediated by a specific assembly factor one would predict such a component to bind nascent CENP-A specifically as well as deliver it to centromeres at the right moment, a requirement not met in an obvious way by any component described, thus far. Two recent reports in *Molecular Cell* and two reports in *Cell* now make clear progress on this problem in fission yeast and human cells, respectively.

Pidoux et al. and Williams et al. identify the *S. pombe* Scm3 homolog of the budding yeast protein of the same name and demonstrate its requirement for CENP-A^{Cnp1} recruitment to centromeres and its direct interaction with CENP-A^{Cnp1}, both in vivo and in vitro (Pidoux et al. 2009; Williams et al. 2009). The interaction between Scm3 and CENP-A^{Cnp1} is dependent on Sim3, suggesting that this histone chaperone may hand over CENP-A^{Cnp1} to Scm3^{Sp} or form a complex with these two proteins (Pidoux et al. 2009). Furthermore, Scm3^{Sp} localizes to the centromere in a cell-cycle-dependent manner, resembling Mis16 and

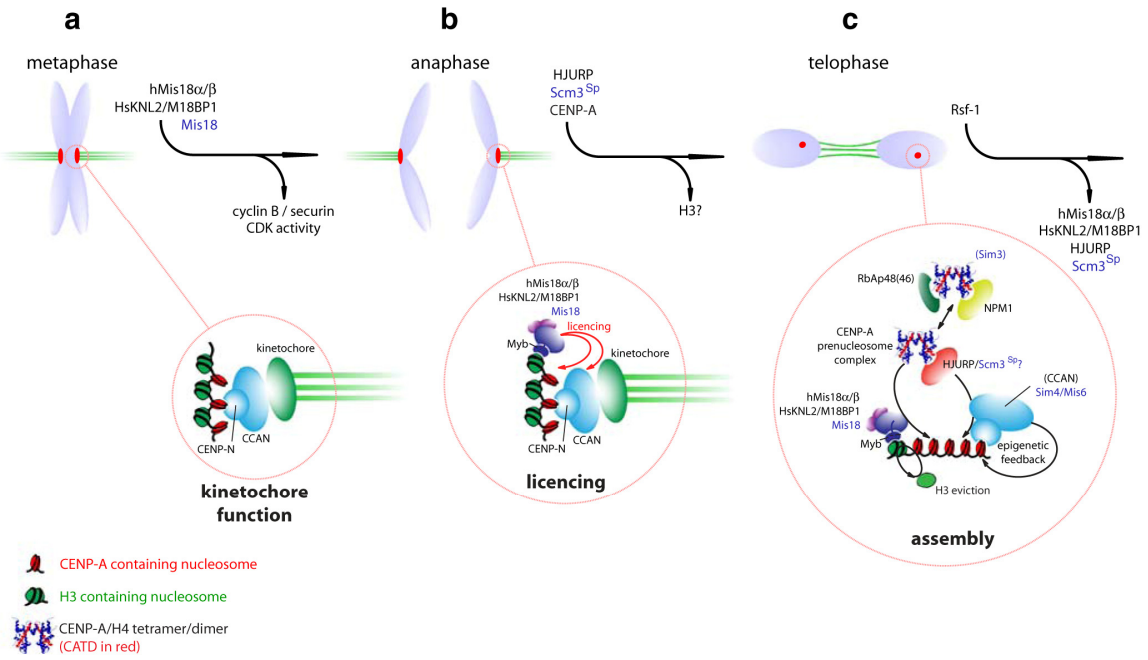

Fig. 2 **a**–**c** Model of the CENP-A assembly process emphasizing temporal control and focusing primarily on the early steps in centromeric chromatin assembly and the role of recently identified components. The human situation is depicted. Functional homology between human and fission yeast proteins (*in blue*) are indicated where appropriate

Mis18 temporal localization consistent with a direct role in CENP-A assembly.

Two independent efforts by Foltz et al. and Dunleavy et al. in human cells identify Holliday junction recognizing protein (HJURP; Kato et al. 2007) as a specific component of the CENP-A prenucleosomal complex but not H3.1 complexes by purification of soluble non-chromatin-bound histone fractions (Dunleavy et al. 2009; Foltz et al. 2009). This complex also includes nucleophosmin (NPM1) and histone H4. While both NPM1 and HJURP were found at centromeric chromatin at low levels in a previous study (Foltz et al. 2006), they are abundant in the CENP-A prenucleosomal complex. HJURP depletion severely impacts nascent CENP-A assembly as shown by SNAP-based pulse labeling and transient expression of GFP-CENP-A. Moreover, HJURP localizes to centromeres during late telophase through early G1, suggesting that it acts as a specific chaperone that can function as a CENP-A targeting factor. HJURP sequences reveal five conserved tryptophan residues similar to the tryptophan-aspartate (WD40) repeats found in other chromatin assembly factors, such as CAF1p60 and HIRA. Consistent with a role as a CENP-A chaperone, HJURP readily binds to CENP-A/H4 in vitro but not to H2A/H2B dimers (Foltz et al. 2009). This binding is mediated through the CATD that is responsible for maintaining centromeric chromatin structure (Foltz et al. 2009). The role of NPM1 in CENP-A assembly is unclear as it has affinity to a broad set of histones. In addition, modest depletions do not affect CENP-A assembly, and it appears to be present in prenucleosomal complexes distinct from those containing HJURP (Dunleavy et al. 2009). Perhaps similar to Sim3 and RbAp48, NPM1 may complex with an inactive pool of nascent CENP-A that transfers CENP-A/H4 to HJURP (Fig. 2c). Intriguingly, it has been previously reported that HJURP can relocalize to nuclear foci upon induction of DNA damage and can bind to holiday junctions in vitro (Kato et al. 2007). This suggests that HJURP may bind DNA directly, perhaps through secondary structures. It will be of interest to determine whether such conditions exist at the centromere region induced either by DNA damage or otherwise.

Functional conservation of the CENP-A assembly process

Although Scm3^{Sp} and HJURP do not display any obvious sequence homology, they appear to play strikingly similar roles in CENP-A assembly. Both Scm3^{Sp} and HJURP are targeted to centromeres following mitotic exit. Scm3^{Sp} stays centromere-localized through the fission yeast S and G2 phases (Pidoux et al. 2009) while HJURP localization

Table 1 Overview of fission yeast and human proteins involved in CENP-A localization or assembly

S. pombe	Human	Centromere localized	CENP-A associated	References
Ams2	–	Central core and inner repeats	Not reported	(Chen et al. 2003; Takahashi et al. 2005)
Hrp1	CHD1[a]	Transiently to inner and outer repeats in *S. pombe*	Not reported	(Walfridsson et al. 2005)
–	Rsf-1	Transient centromere component in mid G1	Yes (CENP-A chromatin)	(Perpelescu et al. 2009)
Sim3	NASP[a]	No	Yes in *S. Pombe*, not reported in human	(Dunleavy et al. 2007)
Sim4	CENP-K	Constitutive centromere component	Indirect	(Okada et al. 2006; Pidoux et al. 2003)
Mis6	CENP-I	Constitutive centromere component	Indirect	(Okada et al. 2006; Takahashi et al. 2000)
Mis15	CENP-N	Constitutive centromere component	Yes in human (nucleosomal CENP-A only)	(Carroll et al. 2009; Foltz et al. 2006; Hayashi et al. 2004; McClelland et al. 2007)
Mis17	CENP-M	Constitutive centromere component	Indirect	(Fukagawa and De Wulf 2009; Hayashi et al. 2004)
Mis12[a]	hMis12	Constitutive but in human cells absent during G1	Indirect	(Cheeseman and Desai 2008; Kline et al. 2006)
Mis16	RbAp46/RbAp48	Transient in fission yeast, absent in human cells.	Yes in human cells and Drosophila	(Dunleavy et al. 2009; Furuyama et al. 2006; Hayashi et al. 2004)
–	M18BP1/HsKNL2	Transient centromere component in early G1	Not detected	(Fujita et al. 2007; Maddox et al. 2007)
Mis18	hMis18α and β	Transient centromere component in early G1	Not detected	(Fujita et al. 2007; Hayashi et al. 2004)
–	HJURP	Transient centromere component in early G1	Yes	(Dunleavy et al. 2009; Foltz et al. 2009)
Scm3	–	Transient centromere component	Yes	(Pidoux et al. 2009; Williams et al. 2009)

Proteins are listed according to their ability to localize to centromeres and/or to bind CENP-A

[a] Ortholog for which no role in CENP-A assembly has been found or reported

occurs only in hMis18α centromere-positive cells in early G1 (Foltz et al. 2009), roughly consistent with the times of CENP-A assembly in the respective organism. Both proteins interact directly with CENP-A. HJURP is part, primarily, of the CENP-A prenucleosomal complex. Whether $Scm3^{Sp}$ binds to soluble prenucleosomal CENP-A has not been directly tested. However, in vitro binding experiments show $Scm3^{Sp}$ binding can occur to recombinant non-nucleosomal CENP-A. Indeed, the budding yeast Scm3 protein forms a trimeric complex with Cse4 and H4 in vitro in striking similarity to the HJURP prenucleosomal complex (Mizuguchi et al. 2007). In addition, both HJURP and $Scm3^{Sp}$ appear to cooperate with RbAp48/46 (Mis16 in the case of $Scm3^{Sp}$). HJURP and RbAp48 can both bind prenucleosomal CENP-A, although they do not appear to be in the same complex (Dunleavy et al. 2009). Nevertheless, depletions of RbAp48 and RbAp46 result in loss of HJURP and consequently in a CENP-A assembly defect suggesting that RbAp48/46 are required for maintaining the prenucleosomal CENP-A assembly complex (Dunleavy et al. 2009). Similarly, $Scm3^{Sp}$ was shown to interact with Mis16 at least transiently and dependents on Mis16 for its centromere localization (Williams et al. 2009). Taken together, at least at a functional level, the HJURP and $Scm3^{Sp}$ proteins appear to have analogous roles in recruiting CENP-A to centromeres. It should be noted though that several homologs of Scm3 carry Myb domains (Aravind et al. 2007). It is, therefore, possible that at least in some fungal species, Scm3 may serve a role reminiscent of the Myb domain containing M18BP1/HsKNL2 protein (discussed below). Current evidence, however, does not favor this possibility since fission yeast Scm3 lacks a Myb domain, and the M18BP1/HsKNL2 protein does not appear to bind to CENP-A.

How does the prenucleosomal loading complex target to the centromere?

$Scm3^{Sp}$ localization at centromeres depends on Mis18 as well as on the constitutive centromere components Sim4 and Mis6 (Pidoux et al. 2009; Williams et al. 2009). Similarly, CENP-A assembly in human cells depends on members of the constitutive centromere complex (CCAN)

as well as the hMis18/HsKNL2 proteins (Carroll et al. 2009; Fujita et al. 2007; Maddox et al. 2007; Okada et al. 2006). What emerges is a model where the CENP-A prenucleosome complex is targeted to centromeres by HJURP in human cells or Scm3 in fission yeast through an interaction either with the CCAN or by binding the Mis18/KNL2 proteins (Fig. 2c). Indeed, in support of the latter possibility, $Scm3^{Sp}$ has been shown to interact with Mis18 both by pull downs as well as by in vitro assays providing a molecular link between CENP-A and the Mis18 proteins that was, thus far, lacking (Pidoux et al. 2009). The extended question becomes how the Mis18/KNL2 proteins are targeted to the centromere themselves. Intriguingly, the human hMis18/HsKNL2 proteins arrive at the centromere before CENP-A assembly (Fig. 1; Fujita et al. 2007; Jansen et al. 2007; Maddox et al. 2007) in a manner that is largely unaffected by severe depletions of CENP-A chromatin (Fujita et al. 2007; Hayashi et al. 2004). Consistent with this, the M18BP1/HsKNL2 protein sports a divergent Myb/SANT domain suggesting the complex may be targeted directly to DNA or histone tails (Boyer et al. 2004). Although complete loss of CENP-A may ultimately lead to complete centromere disruption, these experiments suggest that the Mis18/KNL2 proteins target to centromeres distal from CENP-A nucleosomes and "license" the centromere for recruitment of new CENP-A nucleosomes that ensues shortly thereafter (Fig. 2b). The term "licensing" or "priming" refers here to some action at a distance fuelled largely by the lack of any detectable interaction between (h)Mis18/HsKNL2 and CENP-A. Fission yeast mis18 (and mis16) mutants display elevated H3 and H4 acetylation levels specifically in the central core where $CENP\text{-}A^{Cnp1}$ binds (Hayashi et al. 2004). In human cells, the consequences of hMis18α depletion can be alleviated by experimentally increasing global acetylation levels (Fujita et al. 2007). Although, at first glance, the fission yeast and human cell experiments appear to give exactly opposite results, acetylation of an as of yet unknown target may be involved in centromere licensing for CENP-A assembly. The target for licensing may be centromeric nucleosome components or member of the CCAN which would in turn allow for recruitment of the CENP-A/HJURP prenucleosomal complex (Fig. 2b, c).

How the HJURP-CENP-A/H4 complex targets to the centromere in human G1 cells remains to be tested. It is tempting to speculate that analogous to fission yeast the hMis18/HsKNL2 proteins may recruit HJURP directly to the centromere. Alternatively, HJURP binds a centromere compartment that is modified through hMis18/HsKNL2 licensing. Members of the CCAN that bind CENP-A through CENP-N and have been shown to affect CENP-A assembly would be obvious candidates. In this way, the epigenetic feedback loop would be closed explaining how epigenetic centromere identity provided by the CENP-A nucleosome is transferred to the next generation of nucleosomes set to survive into subsequent cell divisions. Further in vitro binding studies reminiscent of those conducted by Carroll and colleagues for CENP-A nucleosomes (Carroll et al. 2009) are bound to shed light on this issue.

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Appendix 4 – Cdk Activity Couples Epigenetic Centromere Inheritance to Cell Cycle Progression

Developmental Cell
Article

Cdk Activity Couples Epigenetic Centromere Inheritance to Cell Cycle Progression

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SUMMARY

Centromeres form the site of chromosome attachment to microtubules during mitosis. Identity of these loci is maintained epigenetically by nucleosomes containing the histone H3 variant CENP-A. Propagation of CENP-A chromatin is uncoupled from DNA replication initiating only during mitotic exit. We now demonstrate that inhibition of Cdk1 and Cdk2 activities is sufficient to trigger CENP-A assembly throughout the cell cycle in a manner dependent on the canonical CENP-A assembly machinery. We further show that the key CENP-A assembly factor Mis18BP1^{HsKNL2} is phosphorylated in a cell cycle-dependent manner that controls its centromere localization during mitotic exit. These results strongly support a model in which the CENP-A assembly machinery is poised for activation throughout the cell cycle but kept in an inactive noncentromeric state by Cdk activity during S, G2, and M phases. Alleviation of this inhibition in G1 phase ensures tight coupling between DNA replication, cell division, and subsequent centromere maturation.

INTRODUCTION

Accurate segregation of newly replicated chromosomes during mitosis is essential for the maintenance of genome integrity. Central to preserving fidelity of this process is the kinetochore, which forms the chromosomal attachment site for spindle microtubules and is required for chromosome movement and mitotic checkpoint signaling (Cheeseman and Desai, 2008). The centromere is a unique constitutive chromatin domain that assembles the kinetochore during mitosis and is essential for mitotic progression (Allshire and Karpen, 2008). Centromeres are propagated epigenetically, largely independent of any particular DNA sequence (Warburton et al., 1997; Vafa and Sullivan, 1997; Warburton, 2004). Key to the structure and maintenance of the centromere is the specific assembly of the histone H3 variant Centromere Protein A (CENP-A) into centromeric nucleosomes (Silva and Jansen, 2009; Olszak et al., 2011; Barnhart et al., 2011; Guse et al., 2011). Inheritance and replication of this mark is essential to ensure epigenetic propagation of centromere identity (Olszak et al., 2011; Barnhart et al., 2011). Indeed, CENP-A containing nucleosomes are extremely stable and maintained throughout the cell cycle, being redistributed only during S phase (Jansen et al., 2007; Hemmerich et al., 2008; Shelby et al., 2000; Régnier et al., 2005). Importantly, centromeric chromatin replication is uncoupled from centromeric DNA replication and, at least in metazoans, restricted to late mitosis/early G1 phase of the cell cycle (Jansen et al., 2007; Hemmerich et al., 2008; Schuh et al., 2007; Bernad et al., 2011; Moree et al., 2011). Assembly strictly depends on passage through mitosis (Jansen et al., 2007; Schuh et al., 2007; Bernad et al., 2011; Moree et al., 2011), which ensures tight coupling of centromere duplication to cell cycle progression. However, the mitotic trigger that initiates centromere propagation has not been identified. Possible candidates for this have been previously proposed (Figure 1A), including changes in nuclear architecture (Jansen et al., 2007), anaphase promoting complex/cyclosome (APC/C)-mediated destruction of a specific inhibitor of CENP-A assembly (Erhardt et al., 2008), or assembly of a proper kinetochore-microtubule interface (Mellone and Allshire, 2003; Jansen et al., 2007; Allshire and Karpen, 2008).

Here, we sought to identify the molecular signal that temporally controls CENP-A assembly. We find that inhibiting Cdk1 and Cdk2 in any phase of the cell cycle is sufficient to trigger rapid CENP-A assembly in a canonical fashion. Thus, our results point to a simple mechanism that excludes the need for any active involvement of mitosis in subsequent CENP-A assembly, other than the concomitant downregulation of Cdk activity upon mitotic exit.

RESULTS

Cdk Inhibition Triggers CENP-A Assembly prior to Mitosis

In order to identify the molecular mechanism controlling the unusual timing of CENP-A assembly, we employed SNAP-based fluorescent quench-chase-pulse labeling (see Experimental Procedures and Jansen et al., 2007) to uniquely and directly track the fate of nascent proteins. One defining feature of mitotic exit is APC/C-mediated destruction of cyclin B and concomitant loss of associated cyclin-dependent kinase (Cdk) activity. We therefore hypothesized that the CENP-A assembly process

might be controlled directly by Cdk activity, without a strict need for APC/C activation, destruction of APC/C targets apart from the Cdk activator cyclin B, or any other aspect of mitosis. To test this hypothesis, we synchronized HeLa cells in G2 phase, upon which a nascent pool of CENP-A-SNAP, synthesized during the preceding S phase, was fluorescently pulse labeled. A brief (1 hr) treatment with the pan-Cdk inhibitors Roscovitine or Purvalanol A induced CENP-A assembly into the centromere in nearly half the cyclin B positive (G2 phase) population, whereas assembly is never observed in control cells at this stage in which the nascent pool remained diffusely nuclear (Figures 1B–1E; see Figures S1A and S1B available online). We confirmed these results in nontransformed, hTERT immortalized RPE cells, without thymidine-mediated cell synchronization (Figure S2). Assembly under transient Roscovitine-treated conditions in G2 phase resulted in stable incorporation of CENP-A into chromatin, as it is retained at centromeres on condensed mitotic chromosomes following Roscovitine washout (Figure S1F). However, a brief, 1 hr induction of CENP-A assembly in this phase is likely incomplete as normal CENP-A accumulation at centromeres continues for the duration of G1 phase (~10 hr) (Lagana et al., 2010).

Roscovitine treatment of G2 cells did not change cell cycle position as treatment prevents mitotic entry and cyclin B levels remained high (Figures 1B and 1E; Figure S1C). Preservation of high cyclin B levels suggests that Roscovitine-treated cells do not enter a precocious G1-like state by premature activation of APC/C-mediated protein destruction. Indeed, treatment of cells with the proteasome inhibitor MG132 or protein synthesis inhibitor Cycloheximide did not interfere with G2-induced CENP-A assembly. Therefore, the destruction of a specific CENP-A assembly inhibitor or de novo synthesis of an assembly factor is unlikely to be required for CENP-A loading (Figures 1E and 1F; Figures S1D and S1E). These results suggest that the CENP-A assembly machinery is present and poised for activation prior to mitosis. In addition, they argue against a role for APC/C-mediated destruction of a putative inhibitor of CENP-A assembly other than the Cdk activator cyclin B.

Cdk1 and Cdk2 Are Sufficient to Maintain Cell Cycle Control of CENP-A Assembly

Our small molecule inhibitor experiments indicate that, prior to mitosis, the CENP-A assembly machinery is present but kept in an inactive state by Cdk activity. The predominant Cdks that are active during G2 phase and mitosis are Cdk2 and Cdk1, respectively. Both kinases are naturally deactivated upon mitotic entry and mitotic exit, respectively (Pines, 2006), and both are strongly inhibited by Roscovitine and Purvalanol A (Wesierska-Gadek and Krystof, 2009). However, due to the broad substrate specificity of these inhibitors (Wesierska-Gadek and Krystof, 2009), we cannot determine which Cdk (if any) is responsible for controlling CENP-A assembly. To address this directly, we turned to chicken DT40 cells that harbor defined mutations in Cdk1 and/or Cdk2. We utilized cells that carry either a homozygous *CDK2* deletion (*cdk2*$^{-/-}$) and/or express analog-sensitive Cdk1 in a homozygous *cdk1* null background (*cdk1as*) (Hochegger et al., 2007). Cdk1as can be selectively inhibited by addition of the ATP analog 1NM-PP1. 1NM-PP1 does not affect cell cycle progression of wild-type DT40 cells, underscoring the specificity to the *cdk1as* mutation (data not shown and Hochegger et al., 2007). These cells were further retrofitted to stably express subendogenous levels of SNAP-tagged chicken CENP-A, which results in a centromeric fluorescent signal following pulse labeling with TMR-*Star* (Figure 2A and 2A′).

Assembly of nascent SNAP-CENP-A in DT40 cells occurred only in cells with low cyclin B2 levels and can be fully blocked when cells are prevented from entry into G1 by nocodazole-induced mitotic checkpoint arrest (Figures 2A–2B). This demonstrates that, like in human cells (Figure 1B) (Jansen et al., 2007; Hemmerich et al., 2008), *Drosophila* embryos (Schuh et al., 2007) and *Xenopus* extracts (Bernad et al., 2011; Moree et al., 2011), chicken DT40 cells assemble CENP-A at centromeres only upon mitotic exit. Cdk2 protein is nonessential in DT40 cells (Hochegger et al., 2007) and mice (Berthet et al., 2003; Ortega et al., 2003), possibly due to compensation by cyclin A- and E-mediated Cdk1 activity (Hochegger et al., 2007; Santamaría et al., 2007). Consistently, timing of assembly did not change in the *cdk2*$^{-/-}$ mutant background (Figures 2A and 2B), indicating that inhibition of Cdk2 alone is not sufficient to induce unscheduled CENP-A assembly. To test for the involvement of Cdk1 we synchronized *cdk1as* single or *cdk1as/cdk2*$^{-/-}$ double mutants in low (1 μM) levels of 1NM-PP1. At this concentration Cdk1 activity is sufficient to drive S phase progression but not high enough to allow entry into mitosis, resulting in a G2 arrest (Figure S3A) (Hochegger et al., 2007). These cells were either maintained in G2 in low inhibitor concentrations, or released into G1 phase by inhibitor removal, or shifted to high (10 μM) doses of ATP analog to completely abolish Cdk1 activity (Figure 2C). While centromeric CENP-A assembly was detected in G1 phase in cells of either genotype, *cdk1as* single mutants showed little centromere assembly in G2 (Figures 2C′ and 2D). Strikingly, G2 arrested *cdk1as/cdk2*$^{-/-}$ double mutants readily incorporated CENP-A at centromeres in virtually all cells in both low and high 1NM-PP1 concentrations (Figures 2C and 2D). These results were confirmed by pan-Cdk inhibition using Roscovitine, consistent with our observations in HeLa cells (Figure S3D). Clearly, loss of both Cdk1 and Cdk2 activities is necessary and sufficient to trigger premature CENP-A loading, which indicates that these are responsible for suppressing the CENP-A assembly machinery prior to mitotic exit.

CENP-A Assembly Can Be Induced in S Phase

We next determined whether CENP-A assembly, induced by the loss of Cdk activity, was restricted to G2 phase. Randomly cycling DT40 cells spend up to 60% of their time in S phase (Zhao et al., 2007). However, Cdk1/2 double inactivation in these cells resulted in CENP-A assembly in ~78% of the population (Figures 3A and 3A′; Figure S3B) suggesting that CENP-A loading can be induced also in S phase cells. To directly test this possibility, we synchronized both *cdk1as* and *cdk1as/cdk2*$^{-/-}$ cells in S phase with Hydroxyurea (HU) and assayed centromere assembly of a nascent CENP-A-SNAP pool during HU arrest. In the presence of Cdk2 activity little or no CENP-A assembly was observed, even when Cdk1 was fully inhibited (Figures 3B and 3B′; Figure S3C) indicating that Cdk2 is sufficient to block CENP-A assembly. However, in the absence of Cdk2 ~37% of the cells with active Cdk1 (no 1NM-PP1) assembled

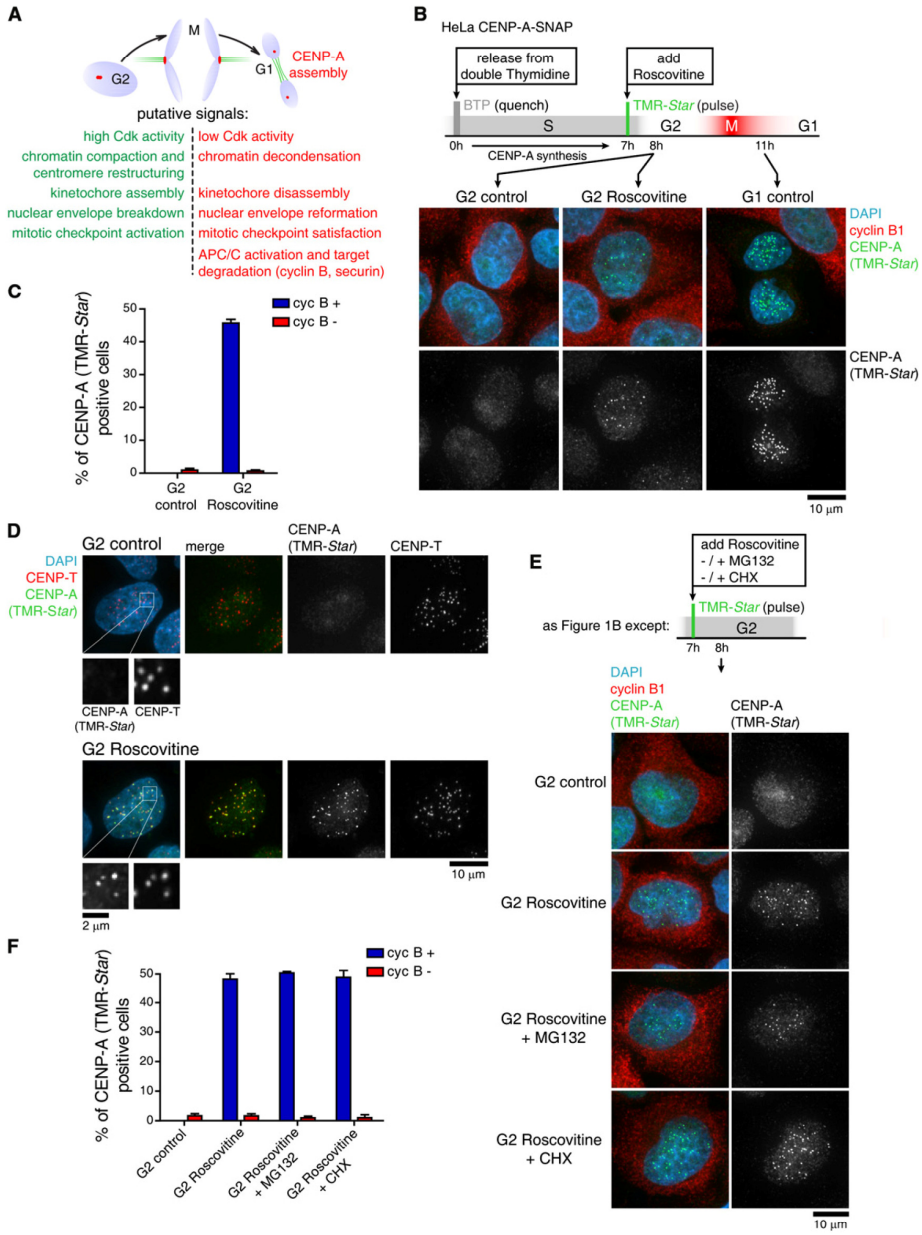

Figure 1. Cdk Inhibition Induces Premature CENP-A Assembly at Centromeres

(A) Schematic representing mitotic passage with key steps during either mitotic entry (green) or mitotic exit (red) that are potential signals for subsequent CENP-A assembly in early G1.

(B) HeLa CENP-A-SNAP cells were synchronized at the G1/S boundary by double thymidine arrest. S phase synthesized CENP-A-SNAP was subsequently pulse labeled in G2 phase, 7 hr after release from thymidine. G2 cells were mock treated (G2 control), treated with Roscovitine for 1 hr, or allowed to cycle through mitosis (G1 control) prior to fixation. Cells were counterstained for cyclin B and with DAPI to indicate G2 status and DNA, respectively.

(C) Quantification of (B).

CENP-A, which increased to ~80 when Cdk1 was further inhibited (10 μM 1NM-PP1), indicating that Cdk1 can contribute but is not sufficient to prevent CENP-A assembly in S phase. These results demonstrate that cells arrested in S phase are competent for CENP-A assembly and that assembly at this stage is prevented primarily by Cdk2 activity. This is consistent with recent findings highlighting nonredundant roles for Cdk2 in cell cycle progression (Merrick et al., 2008, 2011).

Cells held in S phase by HU treatment are not actively replicating DNA due to HU-induced stalling of DNA replication. To test whether CENP-A assembly can occur in actively replicating cells we synchronized *cdk1as/cdk2*$^{-/-}$ cells in early S phase by HU treatment followed by release in the presence of BrdU to mark actively replicating cells. A nascent CENP-A-SNAP pool was synthesized and labeled during progression through S phase in the presence or absence of 10 μM 1NM-PP1. Three hours following the release from HU, cells were scored for CENP-A assembly and cell cycle position. Upon inhibition of Cdk2 and Cdk1, 85% of cells assembled nascent CENP-A at centromeres under conditions where 70% are in S phase and BrdU positive (Figures 3C and 3C′). These results demonstrate that cells undergoing active DNA replication are competent for CENP-A assembly.

Unscheduled CENP-A Assembly Requires the Canonical Assembly Factors

We next determined whether unscheduled CENP-A assembly induced prior to mitosis resulted from the activation of the canonical CENP-A assembly pathway. To address this, we returned to the HeLa cell system. Mis18α, Mis18β, and the Myb-domain containing protein Mis18BP1^{HsKNL2} (collectively named the Mis18 complex) are essential for CENP-A assembly in G1 phase and are recruited to centromeres during anaphase, just prior to the onset of CENP-A assembly (Silva and Jansen, 2009; Fujita et al., 2007; Maddox et al., 2007). Strikingly, inhibition of Cdk activity in G2 cells resulted in rapid recruitment of GFP-Mis18α and Mis18BP1^{HsKNL2} to centromeres (Figures 4A–4D). Cells displayed either centromere localized GFP-Mis18α alone (Figure 4A, red arrow) or both GFP-Mis18α and nascent CENP-A-SNAP (Figure 4A, green arrow) but never CENP-A-SNAP alone (Figure 4B). This suggests that, as for canonical G1 loading of CENP-A, Mis18α arrives prior to, and is required for subsequent CENP-A assembly under induced conditions in G2 phase. Consistently, siRNA-mediated depletion of Mis18α, Mis18BP1^{HsKNL2}, or the CENP-A-specific chaperone HJURP (Dunleavy et al., 2009; Foltz et al., 2009) resulted in a reduction of both G1 phase assembly of nascent CENP-A as well as unscheduled assembly in G2 phase (Figure 4E). Note that partial depletion of assembly factors under these conditions (to or below 50% of unperturbed levels; Figure S4) is sufficient to impair CENP-A assembly, indicating that these are rate limiting for assembly in both G1 phase and G2 phase.

Molecular Mechanism Maintaining Cell Cycle Control of CENP-A Assembly

Our results thus far identify two upstream cell cycle control kinases that maintain the CENP-A assembly machinery in an inactive state. Aurora B is a mitotic kinase downstream of Cdk activity that is responsible for pericentric H3 phosphorylation from late S through G2 phase into mitosis (Monier et al., 2007) and phosphorylates CENP-A during mitosis (Zeitlin et al., 2001), potentially implicating Aurora B in maintaining cell cycle control of CENP-A assembly. However, treatment of HeLa cells expressing CENP-A-SNAP with inhibitors of either Aurora A or Aurora B (Hoar et al., 2007; Ditchfield et al., 2003) did not alter the G1 phase timing of CENP-A assembly (Figure S5A) nor did they significantly block Roscovitine-induced CENP-A assembly in G2 phase (Figure S5B). These observations suggest that the Aurora kinases are unlikely to be involved in cell cycle control of CENP-A assembly.

Rapid and early recruitment of members of the Mis18 complex suggests that Cdk activity acts either directly or indirectly on these components. Indeed, previous phosphoproteome screens have found that Mis18α, Mis18β, and Mis18BP1^{HsKNL2} are phosphorylated on at least 1, 4, and 20 positions, respectively, including Cdk consensus sites in the latter (Olsen et al., 2006; Dephoure et al., 2008; Wang et al., 2008; Mayya et al., 2009). Mutation of all known S/T phosphorylation sites in Mis18α or Mis18β had no discernible effect on centromere localization (not shown). Strikingly, however, conversion of 24 S/T sites in Mis18BP1^{HsKNL2} to alanine (20 known sites plus an additional 4 S/T residues immediately adjacent to known phospho sites; Mis18BP1^{HsKNL2}-Ala^{24}; Figure 5A) resulted in a precocious recruitment of this protein to centromeres in G2 phase and mitotic cells (Figures 5B and 5C). We find Mis18BP1^{HsKNL2} to be phosphorylated in a cell cycle-dependent manner peaking in cells enriched in mitosis (Figure 5D). Treatment of phosphorylated protein by phosphatase in vitro or by mutation of known sites (Mis18BP1^{HsKNL2}-Ala^{24}) resulted in the loss of high molecular weight species, indicating that most, if not all, phosphorylation events are removed in this mutant (Figure 5D). These results strongly suggest that Mis18BP1^{HsKNL2} is kept in a noncentromeric state by phosphorylation, a state alleviated by loss of phosphorylation during mitotic exit upon diminishment of Cdk activity. While Mis18BP1^{HsKNL2}-Ala^{24} targeting did not result in premature CENP-A assembly (not shown), our results indicate that Cdk-mediated inhibition of CENP-A assembly is likely exerted through controlling centromere localization of key CENP-A assembly factors, including Mis18BP1^{HsKNL2}.

DISCUSSION

Our combined results demonstrate that the CENP-A assembly machinery is present and poised for activity throughout most of the cell cycle, but is kept in an inactive state by Cdk1 and Cdk2 activities until after completion of DNA replication and

(D) Nascent CENP-A-SNAP colocalizes with centromeres (CENP-T) after Roscovitine-induced assembly in G2 phase HeLa CENP-A-SNAP cells.

(E) Experiment as in (B) but with the inclusion of MG132 or cycloheximide (CHX) to block proteolysis or protein synthesis, respectively (see also Figures S1C–S1E).

(F) Quantification of (E). Mean and standard error of the mean (SEM) of three replicates are shown in (C) and (F). Percentage of total cells positive for centromeric CENP-A-SNAP (TMR-*Star*) signal was scored and represented according to cyclin B status. See also Figures S1 and S2.

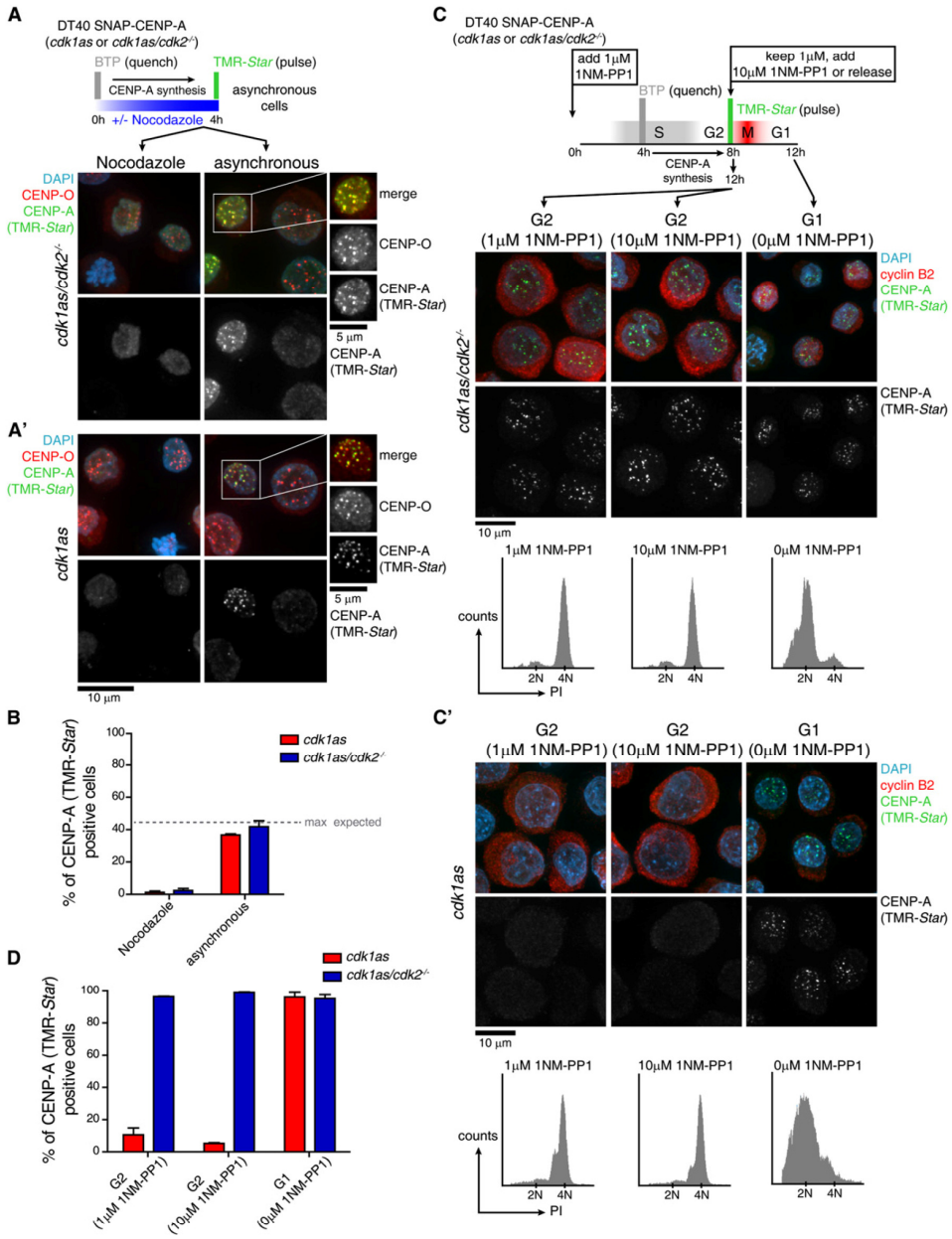

Figure 2. CENP-A Assembly Is Suppressed by Cdk1 and Cdk2 Activity

(A) DT40 *cdk1as/cdk2*$^{-/-}$ double mutant cells (i.e., Cdk2 null but active Cdk1) stably expressing SNAP-CENP-A were assayed for assembly of a nascent pulse labeled pool in either asynchronous cultures or cells prevented from entering G1 phase by nocodazole treatment. Insets show nascent SNAP-CENP-A (TMR-*Star*) colocalization with centromeres (CENP-O).

(A′) as A but for *cdk1as* single mutant (i.e., active Cdk1 and Cdk2).

(B) Quantification of (A) and (A′). Dotted line represents the maximally expected percentage of cells assembling CENP-A (3.5 hr synthesis/8 hr cell cycle [Zhao et al., 2007] × 100 = 44%).

chromosome segregation (Figure 5E). We further demonstrate that the only aspect of mitotic passage that is essential for subsequent CENP-A assembly is the resulting loss of mitotic Cdk activity. This does not exclude the possibility that other aspects of mitosis fine-tune CENP-A homeostasis as previously suggested (Brown and Xu, 2009). Because APC/C-mediated destruction of proteins, other than the Cdk1 activator cyclin B, is not required for CENP-A assembly we argue that the trigger initiating assembly likely depends directly on a phosphoswitch mediated by overlapping Cdk1 and Cdk2 kinase activity. The requirement for exit from mitosis to trigger CENP-A assembly is broadly conserved across transformed and untransformed human cells (Figure 1B; Figure S2) (Jansen et al., 2007; Hemmerich et al., 2008), *Drosophila* embryos (Schuh et al., 2007), and *Xenopus* extracts (Bernad et al., 2011; Moree et al., 2011), implying cell cycle control is maintained in an analogous manner. Intriguingly, however, a recent study demonstrated that unlike *Drosophila* embryos, CID^{CENP-A} assembly in fly cell lines occurs in metaphase (Mellone et al., 2011). Possibly, in these cells, CID assembly is held in check exclusively by cyclin A-mediated Cdk activity during S and G2 phases. Cyclin A is degraded in early mitosis (den Elzen and Pines, 2001; Geley et al., 2001), potentially triggering the centromere targeting of CID in this system.

Phosphorylation of $Mis18BP1^{HsKNL2}$ is inversely correlated with its centromere localization as previously observed for the fission yeast CENP-A assembly factor Smc3 (Pidoux et al., 2009). We show here that preventing phosphorylation is sufficient to drive $Mis18BP1^{HsKNL2}$ centromere location. This suggests that the phosphoswitch controlling CENP-A assembly mediates CENP-A assembly factor activity or localization that includes, but is likely not limited to, $Mis18BP1^{HsKNL2}$.

Two general implications follow from these findings. First, as outlined in Figure 5E, our results provide a logical explanation for the cell cycle coupling between DNA replication, mitosis and CENP-A assembly. CENP-A is redistributed onto sister centromeres in S phase (Jansen et al., 2007; Dunleavy et al., 2011) during which histone H3 is assembled at neighboring positions (Dunleavy et al., 2011). The recently identified CENP-T/CENP-W complex specifically interacts with chromatin containing H3 nucleosomes directly adjacent to CENP-A nucleosomes (Hori et al., 2008; Ribeiro et al., 2010), suggesting that neighboring H3 and CENP-A nucleosomes make up an integral part of the centromeric complex. Importantly, such a mixed H3/CENP-A mitotic chromatin state can be achieved by delaying assembly of CENP-A until after mitosis, providing a possible explanation for the temporal disconnect between DNA replication and CENP-A loading. Second, inhibition of CENP-A loading is mediated exclusively by Cdk1 and Cdk2, which are in turn essential for the initiation of DNA replication (Bell and Dutta, 2002) and entry into mitosis. Conversely, loss of Cdk1 activity triggers licensing of the next round of DNA replication (Mailand and Diffley, 2005) coinciding with the temporal window during which CENP-A assembly is permitted (Figure 5E). We hypothesize that the Cdk-mediated molecular switch that turns the CENP-A assembly machinery "ON" in early G1 and then "OFF" after S phase entry is one and same. Consistent with this notion, quantitative live cell measurements have recently shown that CENP-A assembly in human cells continues until ~10 hr after anaphase, which coincides with late G1/early S phase (Lagana et al., 2010). This temporal restriction of CENP-A assembly activity during G1 phase may represent an important mechanism to maintain a proper centromere size and architecture.

EXPERIMENTAL PROCEDURES

Cell Lines and Constructs

HeLa cells and their derivatives were cultured in DMEM medium (GIBCO) supplemented with 10% newborn calf serum (GIBCO) at 37°C 5% CO_2. A HeLa cell line stably expressing both CENP-A–SNAP and LAP-(GFP)-Mis18α was generated using the previous established stable cell line expressing CENP-A-SNAP (Jansen et al., 2007). A construct containing *LAP-(GFP)-Mis18α* (a gift from I. Cheeseman, MIT, Cambridge, MA) was stably integrated into this cell line via Moloney murine leukemia retroviral delivery. Cells stably expressing CENP-A-SNAP and LAP-(GFP)-Mis18α (referred to as GFP-Mis18α throughout this paper) were selected by Blasticidin S (5 μg/ml; Invitrogen) and Puromycin (1.5 μg/ml; Calbiochem) and single-cell sorted by flow cytometry. The resulting monoclonal lines were expanded and selected by fluorescence microscopy. DMEM-F12 (GIBCO) medium supplemented with 10% fetal bovine serum (FBS; GIBCO) and 0.348% Sodium Bicarbonate was used to culture hTERT-RPE cells stably expressing CENP-A-SNAP at 37°C 5%CO_2. This cell line was generated by retroviral delivery of a construct carrying *CENP-A-SNAP-3XHA* (Jansen et al., 2007) and selected by Blasticidin S (10 μg/ml; Invitrogen) analogous to HeLa cell lines described above.

A *3xHA-SNAP-CENP-A* construct named pLJ404 was generated by inserting a PCR-generated fragment carrying the chicken *CENP-A* open reading frame (gift from T. Fukagawa, NIG, Mishima, Japan) flanked by BamHI and XbaI sites into corresponding sites of pSS26m (Covalys) containing an additional triple HA tag at its N terminus. The resulting 3xHA-SNAP-CENP-A fusion protein (referred to as SNAP-CENP-A throughout this paper) was subcloned into p3XFLAG-CMV-14 (Sigma) resulting in pLJ410 (including a STOP codon, excluding FLAG from the ORF). DT40 cell lines were cultured in RPMI1640 medium (GIBCO) supplemented with 50 μM β-mercaptoethanol, 10% FBS and 1% chicken serum (GIBCO) at 39°C 5%CO_2. Stable lines expressing SNAP-CENP-A were created in DT40 *cdk1as* and *cdk1as/cdk2*$^{-/-}$ cells (Hochegger et al., 2007) by electroporation with a Gene Pulser apparatus (Bio-Rad) at 550 V and 25 μF as previously described (Sonoda et al., 1998). Puromycin (0.5 μg/ml, Calbiochem) and Zeocin (500 μg/ml, Invivogen) were used to select *cdk1as* and *cdk1as/cdk2*$^{-/-}$ cell lines, respectively. Clonal lines expressing SNAP-CENP-A at subendogenous levels were selected by fluorescence microscopy after TMR-*Star* labeling and by western blot using an anti-chicken CENP-A antibody (gift from T. Fukagawa, NIG, Mishima, Japan).

The *Mis18BP1*^{HsKNL2} ORF was amplified from cDNA (kind gift from Paul Maddox) by PCR and cloned into the XhoI/EcoRI sites of pIC113 (kind gift from Iain Cheeseman) creating pLJ415 expressing a GFP-TEV-S-tag-$Mis18BP1^{HsKNL2}$ fusion protein. The construct expressing the $Mis18BP1^{HsKNL2}$-Ala^{24} mutant (pLJ451) was identical to pLJ415 except that residues S110, S134, S135, S191, S192, T260, T261, S263, S299, S365, S541, T653, T821, S824, S914, S991, T992, T993, S1004, S1008, S1086, S1087, S1089, and S1104 are mutated to alanine.

(C) DT40 *cdk1as/cdk2*$^{-/-}$ cells were synchronized in G2 phase with a low concentration (1 μM) of 1NM-PP1, followed by synthesis of a nascent SNAP-CENP-A pool. Cells were then either kept arrested in G2 with low (1 μM) or high (10 μM) 1NM-PP1 or released into G1 followed by fixation and processing for imaging or FACS (PI: propidium iodide). Cyclin B2 staining indicates G2 phase.

(C′) As in (C) but for *cdk1as* single mutant cells.

(D) Quantification of (C) and (C′). Mean and SEM of three replicates of each condition are shown in (B) and (D).

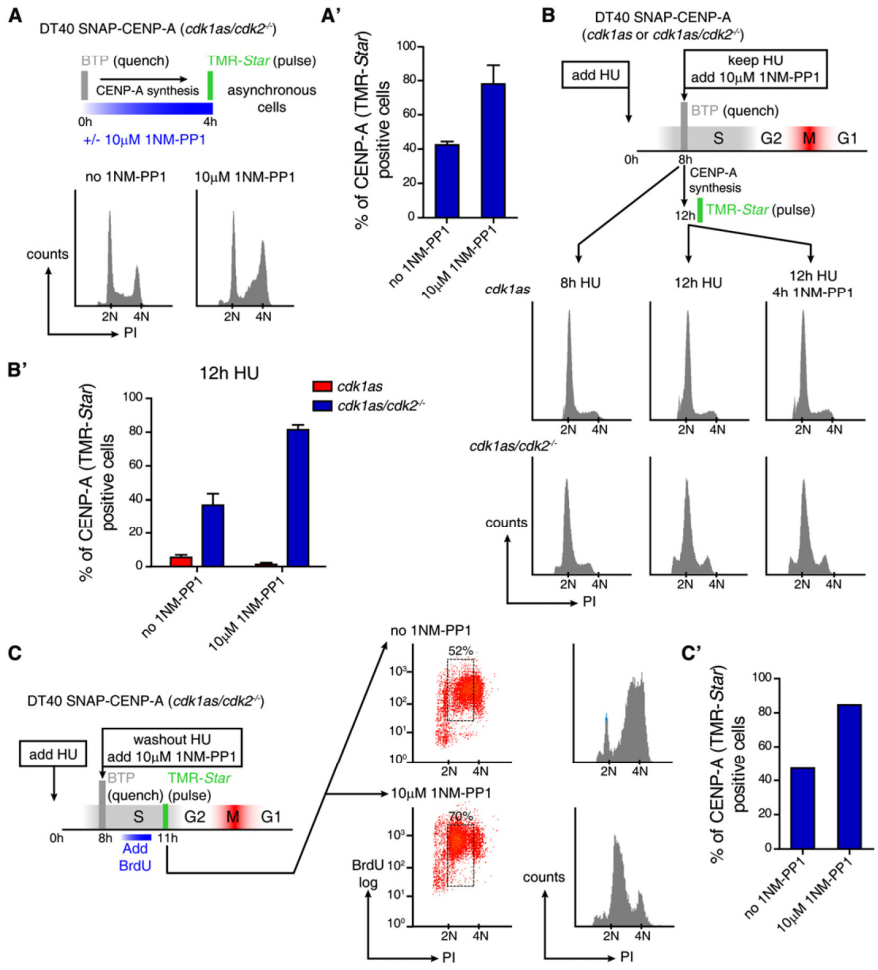

Figure 3. S Phase Cells Are Competent for CENP-A Assembly

(A) Asynchronous cultures of DT40 *cdk1as/cdk2*$^{-/-}$ SNAP-CENP-A cells were treated with DMSO or 10 μM 1NM-PP1 to induce G2 arrest. During the arrest, a nascent pool of SNAP-CENP-A was synthesized and pulse labeled. Cells were then assayed for assembly by microscopy (Figure S3B) and for cell cycle position by FACS. (A′) Percent cells assembling CENP-A at centromeres.

(B) DT40 *cdk1as* and *cdk1as/cdk2*$^{-/-}$ SNAP-CENP-A cells were arrested in S phase by HU treatment. A nascent SNAP-CENP-A pool was labeled in the presence or absence of 1NM-PP1 under continued HU arrest and scored for centromere assembly (Figure S3C). Samples were collected for FACS analysis before (8 hr) and after (12 hr) nascent SNAP-CENP-A synthesis to monitor continued S phase arrest.

(B′) Quantification of B. Percent cells assembling CENP-A at centromeres is scored.

(C) Experiment as in B except that *cdk1as/cdk2*$^{-/-}$ cells were released from HU-induced S phase arrest in the presence of BrdU to monitor active DNA replication during which a new SNAP-CENP-A pool was synthesized. Cells were scored for CENP-A centromere assembly in actively replicating cells in the absence or presence of 1NM-PP1. Fraction of cells undergoing DNA replication is indicated (boxed region). PI: propidium iodide.

(C′) Quantification of percentage of cells assembling CENP-A. Mean and SEM of 3 replicates of each condition are shown in (A′) and (B′). See also Figure S3.

Cell Synchronization, Transfection, and Drug Treatments

HeLa cells were synchronized by a double thymidine block. Cells were treated with thymidine (2 mM, Sigma) for 17 hr, washed twice in medium, and released in medium containing deoxycytidine (24 μM, Sigma) for 9 hr. Subsequently, cells were treated again with thymidine for 16 hr, and finally released into medium containing deoxycytidine and assayed.

HeLa cells were transfected with 250 ng of DNA, 1 μl Plus Reagent and 1.25 μl of lipofectamine (Invitrogen) in Optimem (GIBCO) according to manufactures instructions for Figure 5B. For Figure 5D, HeLa cells were transfected with 400 ng of plasmid DNA using Effectene transfection reagent (QIAGEN) in Opti-MEM reduced serum media (Invitrogen) according to the manufacturer's instructions.

Developmental Cell
Cell Cycle Control of CENP-A Assembly

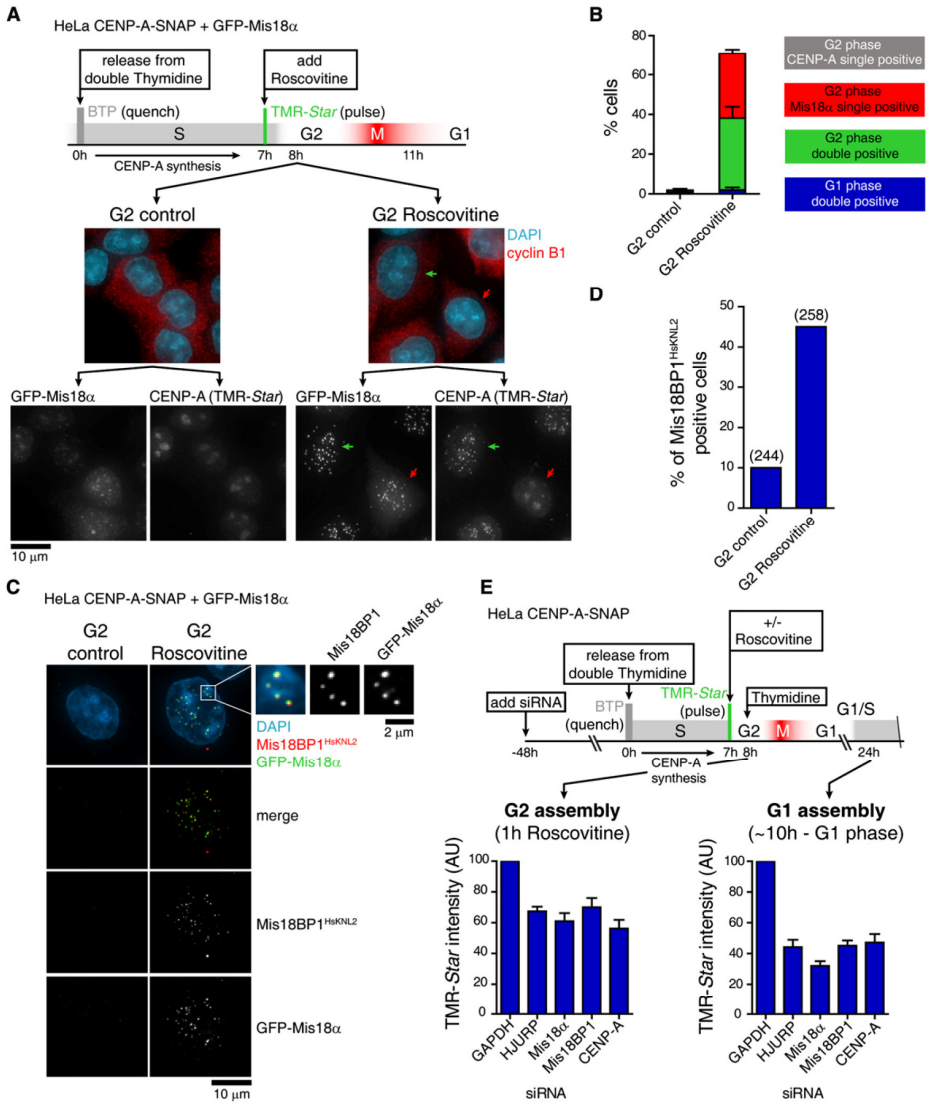

Figure 4. Unscheduled CENP-A Assembly in G2 Phase Occurs through the Canonical Assembly Pathway

(A) CENP-A-SNAP, GFP-Mis18α double-tagged HeLa cells were treated as in Figure 1B. G2 control or Roscovitine-treated cells were counterstained for cyclin B to confirm G2 status and imaged to determine GFP-Mis18α and nascent CENP-A-SNAP centromere localization.

(B) Quantification of (A).

(C) Experiment as in A but stained using antibodies against endogenous Mis18BP1^{HsKNL2}.

(D) Quantification of (C). Number of cells analyzed is indicated between brackets.

(E) HeLa CENP-A-SNAP cells were treated with siRNAs against indicated targets (GAPDH and CENP-A serve as negative and positive controls, respectively), synchronized by double thymidine block combined with SNAP quench-chase-pulse labeling. Cells were treated with Roscovitine for 1 hr in G2 to induce CENP-A assembly or were cycled into the next cell cycle and collected at the next G1/S boundary following canonical CENP-A assembly. Cells were imaged and TMR-*Star* centromere intensity was determined. More than 1,200 centromeres were quantified per condition. Mean and SEM of three replicates of each condition are shown in (B) and (E). See Figure S4 for siRNA efficiency controls.

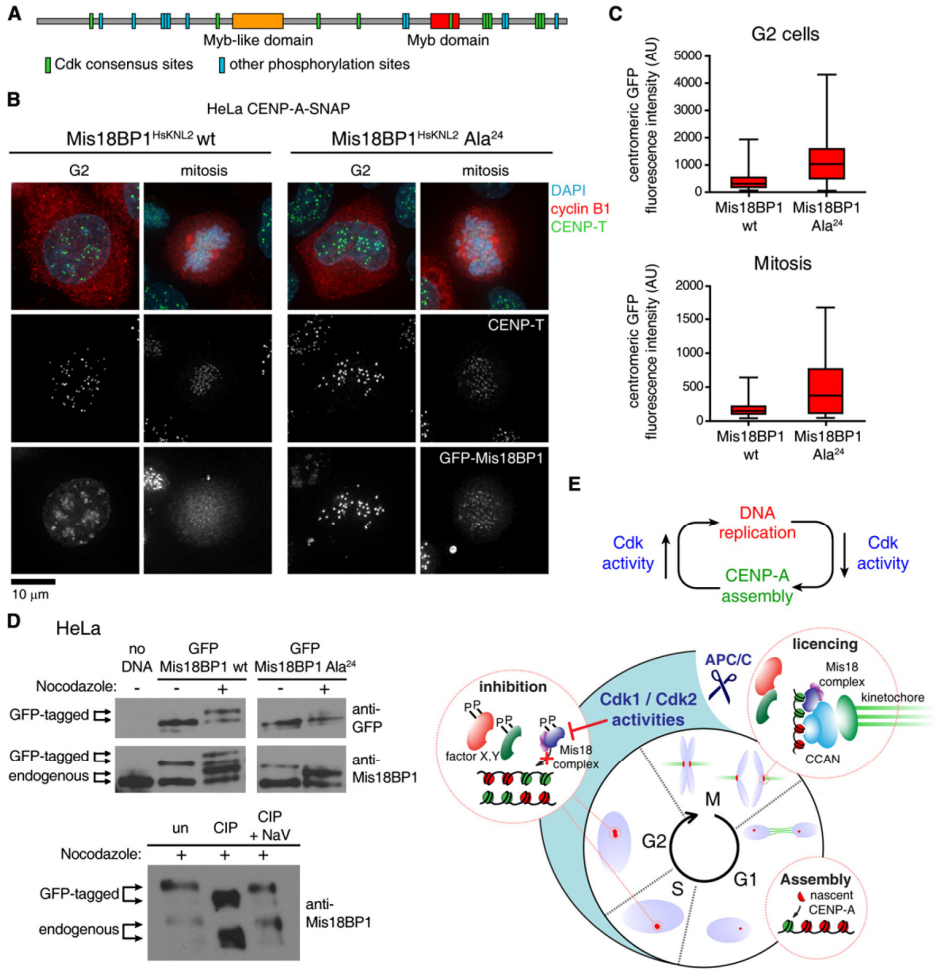

Figure 5. Phosphorylation of Mis18BP1^{HsKNL2} Controls Its Centromere Localization

(A) Schematic of Mis18BP1^{HsKNL2} protein. Relevant domains and known phosphorylation sites are indicated.

(B) Constructs expressing wild-type GFP-tagged Mis18BP1^{HsKNL2} or Mis18BP1^{HsKNL2}-Ala^{24} were transfected into asynchronous HeLa cells 32 hr prior to fixation followed by counterstaining for cyclin B, CENP-T, and with DAPI to indicate G2 status, centromeres and DNA, respectively.

(C) Box and whisker plots of relative GFP-Mis18BP1^{HsKNL2} fluorescent signal per centromere in G2 phase (high cyclin B) and mitotic cells. CENP-T was used as a reference for centromere position. More than 300 and more than 90 centromeres were quantified in G2 and mitotic cells, respectively.

(D) Top: HeLa cells transiently expressing GFP-tagged Mis18BP1^{HsKNL2} or Mis18BP1^{HsKNL2}-Ala^{24} for 29 hr were left untreated or were treated with nocodazole for another 12 hr to enrich for mitotic cells, followed by processing for SDS-PAGE and immunoblotting. Endogenous and GFP-tagged Mis18BP1^{HsKNL2} is detected by indicated antibodies. High molecular weight species are detected in nocodazole treated cells. Bottom: GFP-tagged Mis18BP1^{HsKNL2} was pulled down from mitotic cell extracts that were either untreated (un), treated with calf intestinal alkaline phosphatase (CIP) alone or in combination with sodium orthovanadate (NaV), followed by SDS-PAGE and immunoblotting for Mis18BP1^{HsKNL2}.

(E) Top: Schematic outlining inverse relationship between DNA replication and CENP-A chromatin assembly driven by the Cdk activity cycle. Bottom: Cartoon illustrates Cdk1/Cdk2-mediated inhibition of CENP-A assembly, exerted in part through phosphorylation (P) of Mis18BP1^{HsKNL2} (member of the Mis18 complex) during S, G2, and M phases. Factors X and Y symbolize the involvement of other, yet to be identified, components. Inhibition is alleviated through APC/C-mediated loss of Cdk1 activity in anaphase, targeting the Mis18 complex to the centromere (licensing) followed by CENP-A assembly in G1 phase. Canonical (H3 containing) nucleosomes are shown in green, CENP-A nucleosomes in red. See also Figure S5.

Developmental Cell
Cell Cycle Control of CENP-A Assembly

Roscovitine, Purvalanol A, MG132 and cycloheximide (Sigma) were used at 100 μM, 25 μM, 24 μM, 10 μg/ml, respectively. DMSO (Sigma) was used in control conditions. TNFα (R&D Systems) was used at 50 ng/ml (Seldon et al., 2007). MLN8054 (Selleck Chemicals) and ZM447439 (Enzo Life Sciences) were used at 1 and 2 μM, respectively. DT40 cells were treated with 1 or 10 μM of 1NM-PP1 (synthesized by Chris Larch and Hansjoerg Streicher, Sussex University, UK) for partial or strong inhibition of Cdk1as activity, respectively. DT40 cells were synchronized with 100 ng/ml nocodazole or 2 mM hydroxyurea (HU) (both from Sigma). Following release from an HU-induced S phase arrest (Figure 3C), DT40 cells were allowed to enter S phase for 30 min prior to 1NM-PP1 addition to avoid rearrest due to Cdk1 requirement to enter S phase.

SNAP Quench-Chase-Pulse Labeling

HeLa or hTERT-RPE cells expressing CENP-A-SNAP were pulse labeled by addition of 2 μM BTP (Covalys) in growth medium for 30 min at 37°C, 5% CO_2, for irreversible, nonfluorescent labeling of preexisting SNAP pool. We refer to this step as "quench." Following quenching, cells were chased for 6 hr and 30 min to allow synthesis of new, unlabeled CENP-A-SNAP and were then pulse labeled with 2 μM TMR-*Star* (New England Biolabs) in growth medium for 15 min at 37°C, 5% CO_2, thereby fluorescently labeling the nascent SNAP pool, specifically. DT40 cells were quenched as described for HeLa cells, except that the chase time was 3 hr and 30 min and kept at 39°C 5% CO_2 and the cells were pulse labeled with 5 μM of TMR-*Star*. After each labeling step (both fluorescent and nonfluorescent), cells were washed twice with medium and reincubated at the appropriate temperature to allow excess SNAP substrate to be released from cells. After 30 min, cells were washed again once in medium.

Immunofluorescence

HeLa or hTERT-RPE cells were grown on glass coverslips coated with poly-L-lysine (Sigma) and fixed with 4% formaldehyde (Thermo Scientific) for 10 min. DT40 cells were resuspended in PBS at a concentration of 2 × 10^5 cell/ml and cytospun at 800 rpm during 5 min. Cells were fixed with 4% formaldehyde. HeLa cells were stained with anti-cyclin B1 (1:50; sc-245, Santa Cruz), anti-CENP-T (Barnhart et al., 2011) and anti-Mis18BP1^{HsKNL2} (1 μg/ml; gift from P. Maddox, Université de Montréal, Montreal, Canada). hTERT-RPE cells were stained with anti-α-tubulin (1:2500; clone YL1/2, Serotec), anti-HA (1 μg/ml; clone HA11, Covance). DT40 cells were stained with anti-chicken CENP-O (1:3000; gift from T. Fukagawa, National Institute of Genetics, Mishima, Japan), anti-chicken cyclin B2 (1:50; gift from E. Nigg, University of Basel, Basel, Switzerland). Secondary antibodies (Cy5- or FITC-conjugated anti-mouse, FITC- or Cy3-conjugated anti-rabbit and FITC-conjugated anti-rat) were obtained from Jackson Immunoresearch Laboratories. Dy680 conjugated anti-mouse antibodies were from Rockland Immunochemicals. Cells were stained with DAPI (4′,6-diamidino-2-phenylindole; Sigma) before mounting in Mowiol.

siRNA Transfection

All siRNAs were obtained from Dharmacon. Smart pools were used to deplete Mis18BP1^{HsKNL2}, HJURP, and GAPDH. CENP-A and Mis18α were depleted with siRNAs: 5′-ACAGUCGGCGGAGACAAGGdTdT-3′ and 5′-CAGAAGCU AUCCAAACGUGdTdT-3′, respectively. Sixty picomoles of siRNAs was used for each depletion in a 24-well format according to the manufacturer's instructions.

Flow Cytometry

DT40 cells (10^6) were harvested and fixed during 1 hr at 4°C with 70% ethanol. Cells were washed twice in PBS containing 3% BSA (Sigma) and incubated for 3 hr at room temperature with 5 μg/ml propidium iodide (PI; Sigma) and 200 μg/ml of RNaseA in PBS containing 3% BSA. Subsequent flow-cytometric analysis was performed on a FACScan (Becton Dickinson) or FACS Canto (Becton Dickinson) using CellQuest and FACSDiva software, respectively. For BrdU staining we used an anti-BrdU antibody (347580, Becton and Dickinson). Cells were fixed as described above and processed for staining according to manufactures' instructions. Cells were subsequently stained with a Cy5 secondary antibody from Jackson Immunoresearch and with PI as described above. Cells were analyzed on a CyAn ADP (Beckman Coulter).

Immunoblotting

Extracts of 10^5 (HeLa) or 2 × 10^6 (DT40) cells were separated in a 6% (Figure 5D) or 12% (Figures S1 and S4) SDS-PAGE gel and transferred to a PVDF membrane. Blots were probed with anti-human-cyclin B1 (sc-245, Santa Cruz) at 1:500 dilution, anti-Actin (A2066, Sigma) at 1:1,000 dilution, anti-IκB-α (sc-371, Santa Cruz) at 1:1,000 dilution, anti-HJURP and anti-GFP (Foltz et al., 2009) at 1:2,000 and 1:10,000 dilution, respectively. For blot shown in Figure 5D, anti-GFP was used at 1:1,000 dilution overnight at 4°C. Anti-Mis18BP1$^{hsKNL-2}$ antibody (A302-824A, Bethyl Labs) was a 1:5,000 dilution overnight at 4°C. To screen DT40 monoclonal lines stably expressing subendogenous levels of 3xHA-SNAP-CENP-A we used anti-chicken CENP-A (gift from T. Fukagawa, NIG, Mishima, Japan) and anti-HA (HA11, Covance Research Products, Inc.) antibodies at dilution of 1:3,000 and 1:1,000, respectively. Anti-mouse and anti-rabbit HRP-conjugated secondary antibodies were purchased from Jackson Immunoresearch Laboratories.

Phosphatase Treatment

Lysates were prepared from a HeLa cell line stably expressing LAP-Mis18BP1^{HsKNL2} that had been blocked for 12 hr with 100 ng/ml nocodazole. Cells enriched in mitosis were harvested with 3 mM EDTA-PBS for 10 min at room temperature. Cells were resuspended in buffer containing 75 mM HEPES (pH 7.5), 1.5 mM EGTA, 1.5 mM $MgCl_2$, 150 mM KCl, 15% glycerol, 0.075% IGEPAL, 10 mM imidazole, 200 μM sodium orthovanadate (NaV), 5 mM sodium fluoride, 50 mM β-glycerophosphate, and Complete EDTA-free Protease Cocktail (Roche) and sonicated on ice in 30 s cycles for a total of 2 min. Lysates were centrifuged for 10 min at 1,000 × g at 4°C and the supernatant was passed five times over a column containing His-tagged GFP binding protein (GBP) bound to Ni-NTA agarose. Proteins bound to the GBP beads were washed, resuspended in 30 μl wash buffer and incubated with 10 mM sodium orthovanadate (MP Biomedicals) and/or 60 units of calf intestinal alkaline phosphatase (CIP; New England Biolabs) for 1 hr at 37°C. Reactions were stopped by the addition of SDS sample buffer and separated by SDS-PAGE.

Microscopy

Digital images were captured using a DeltaVision Core system (Applied Precision) that controls an inverted microscope (Olympus, IX-71), coupled to a Cascade2 EMCCD camera (Photometrics). Images (512 × 512) were collected at 1× binning using a 100× oil objective (NA 1.40, UPlanSApo) with 0.2 μm z sections scanning the entire nucleus. Images were subsequently deconvolved and maximum signals were projected as 2D images using softWoRx (Applied Precision). Centromeric TMR intensity was quantified on non-deconvolved, maximum projection images by placing a 7 × 7 pixel square on each centromere using an unrelated centromere marker (CENP-T). Local background corrected intensity values were obtained by subtracting minimum intensity values from maximum values for each centromere measurement.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and can be found with this article online at doi:10.1016/j.devcel.2011.10.014.

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Supplemental Information

Cdk Activity Couples Epigenetic Centromere Inheritance to Cell Cycle Progression

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Supplemental Inventory

Supplemental Figures:

Figure S1, related to Figure 1. Confirmation of results in Figure 1B using the Cdk inhibitor Purvalanol A. Western blots showing cyclin B levels in response to drugs and effectiveness of MG132 and Cycloheximide in preventing protein degradation and synthesis respectively. Roscovitine washout experiments demonstrating stability of G2 assembled CENP-A.

Figure S2, related to Figure 1. Confirmation of key observations shown in Figure 1B in non-transformed human retinal pigment epithelial cells.

Figure S3, related to Figure 3. FACS plots showing cell cycle profiles of mutant DT40 cells in response to 1NM-PP1. Representative images of experiments shown in Figure 3A' and B'. Demonstration of the effect of Roscovitine on CENP-A assembly in DT40 cells.

Figure S4, related to Figure 4. Western blots showing the efficiency of RNAi depletions employed in experiments shown in Figure 4E.

Figure S5, related to Figure 5. Determining the effect of inhibiting Aurora A or Aurora B activity on G1 and G2 phase CENP-A assembly.

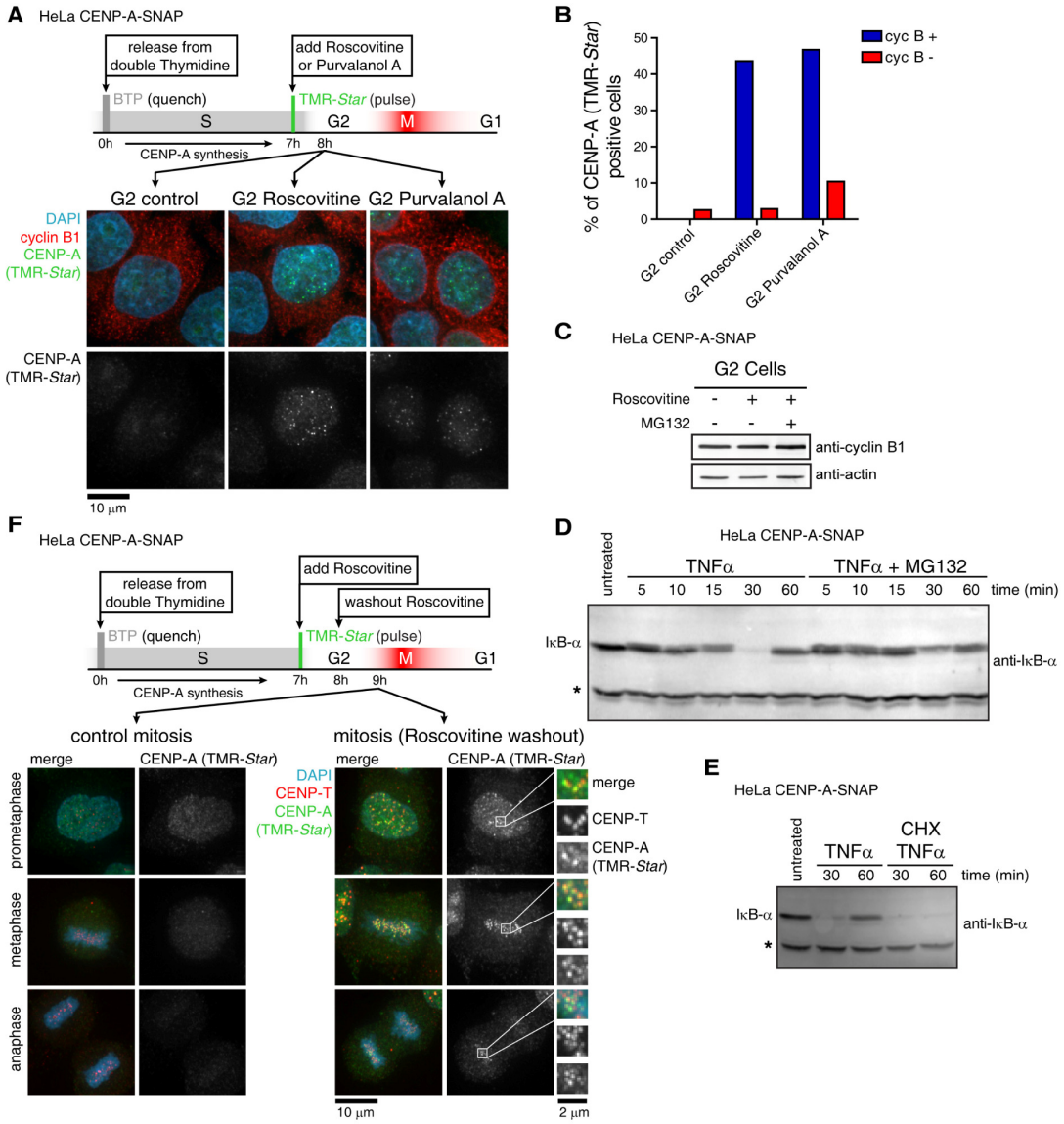

Figure S1. Related to Figure 1. Roscovitine or Purvalanol A treatment induces stable CENP-A-SNAP assembly in G2 phase. (A) Experiment as in Figure 1B, except treatment of cells in G2 with either Roscovitine or Purvalanol A. (B) Quantification of A. (C) G2 phase cyclin B levels remain unchanged after MG132 and/or Roscovitine treatment at conditions used in Figure 1E. HeLa CENP-A-SNAP cells were synchronized in G2 by release from a double Thymidine block followed by a 1 hour treatment with indicated drugs. Cells were processed for immunoblot and probed for cyclin B1 levels or Actin (as a loading control). (D and E) TNFα induces rapid proteolytic degradation of IκB-α which is followed by rapid de novo synthesis (Seldon et al., 2007). MG132 prevents TNFα induced IκB-α degradation while Cycloheximide prevents re-synthesis. HeLa CENP-A-SNAP cells were either treated with TNFα alone, along with MG132 (D) or Cycloheximide (CHX) (E) for up to 60 minutes. IκB-α levels were determined by immunoblot at indicated time points. Asterisk indicates cross reacting band used as a loading control. (F) Experiment as in Figure 1B except cells were released from Roscovitine after a 1 hour induction of CENP-A assembly in G2 phase. Cells were analyzed in mitosis (1 hour after washout) and scored for retention of nascent CENP-A-SNAP on mitotic chromosomes.

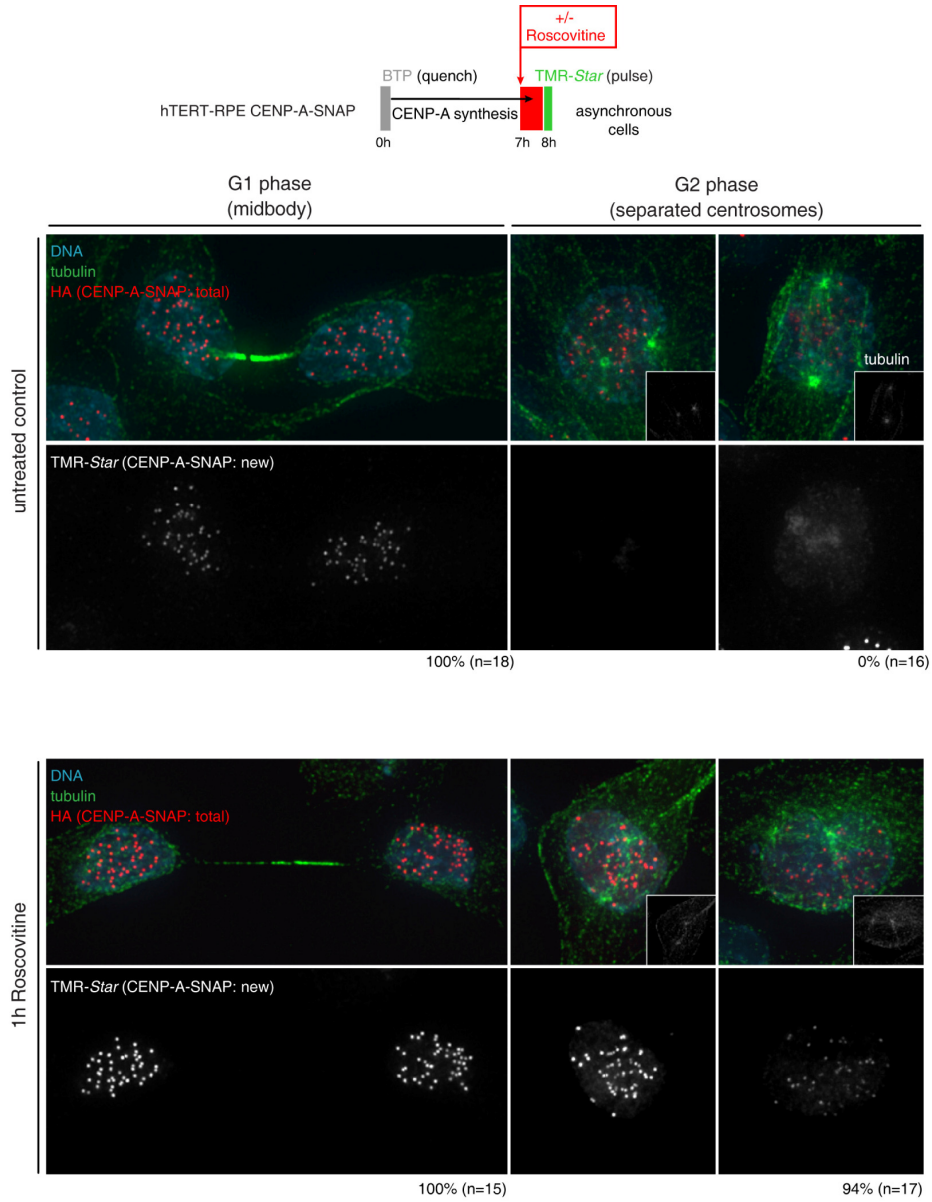

Figure S2. Related to Figure 1. G1 phase assembly of CENP-A-SNAP and G2 induction by Roscovitine in hTERT-RPE cells. Randomly cycling human hTERT-immortalized retinal pigment epithelial cells stably expressing CENP-A-SNAP were subjected to quench-chase-pulse labeling. Newly synthesized fluorescently labeled CENP-A-SNAP (TMR-*Star*) localized to centromeres in early G1 phase cells (as identified by midbody staining) but not to G2 phase cells (marked by separated centrosomes). A one hour treatment with Roscovitine prior to fixation was sufficient to induce CENP-A-SNAP assembly at centromeres in the majority of G2 cells (94%). Two representative images of G2 cells are shown for each condition. Percentage of G1 and G2 cells assembling CENP-A-SNAP is indicated. Cells were counterstained for HA to visualize the total (preincorporated and nascent) pool of CENP-A-SNAP, for tubulin to indicate cell cycle status and with DAPI for DNA.

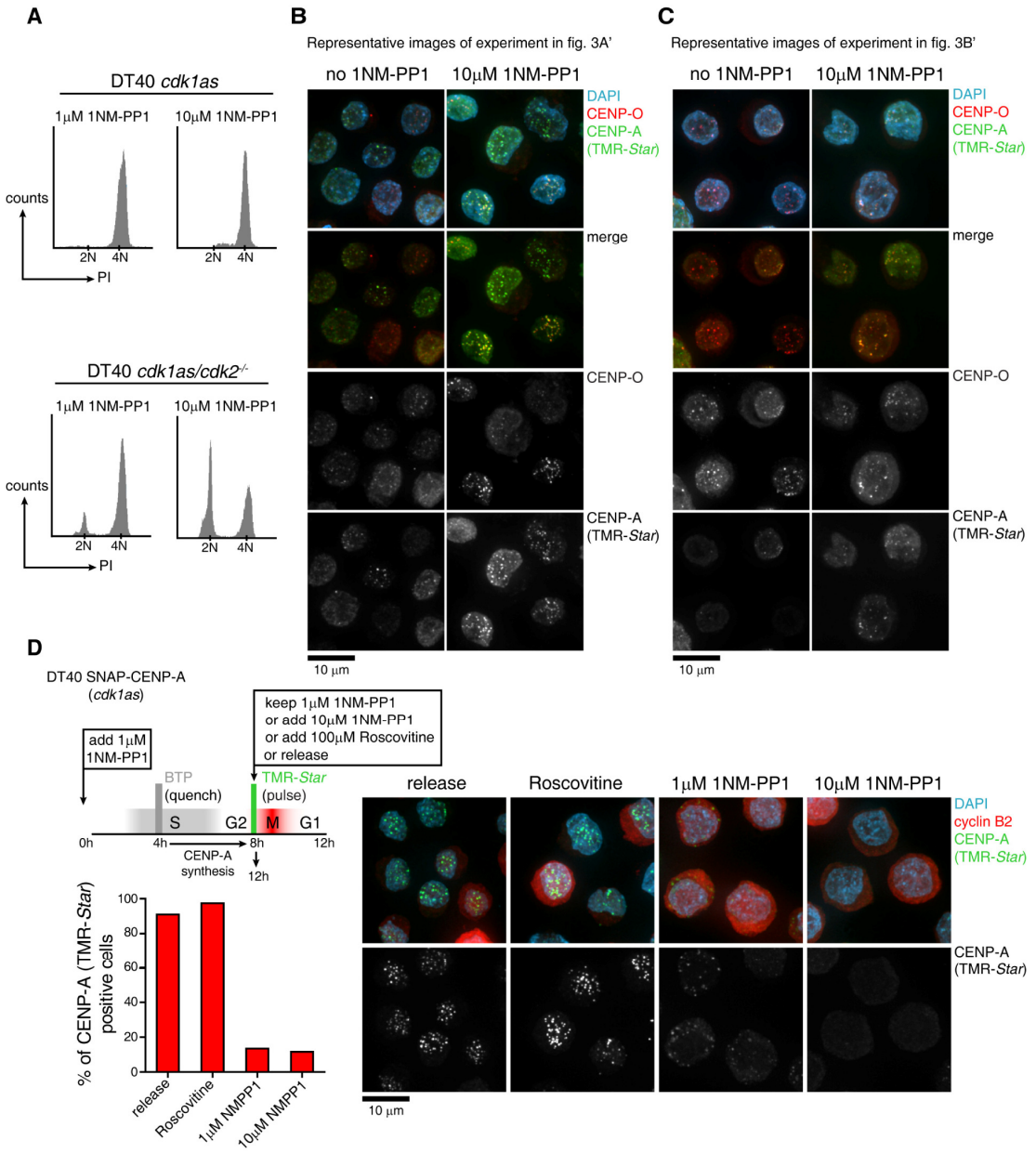

Figure S3. Related to Figure 3. Loss of Cdk1 and 2 activities in DT40 cells results in unscheduled CENP-A assembly. (A) DT40 *cdk1as* or *cdk1as/cdk2*$^{-/-}$ mutant cells were treated with either low (1 μM) or high (10 μM) 1NM-PP1 for 12 hours followed by processing for FACS. Note that *cdk1as* single mutants arrest in G2 at both 1NM-PP1 concentrations used. In contrast, *cdk1as/cdk2*$^{-/-}$ double mutants can enter S phase and progress to G2 in low 1NM-PP1 concentration whereas high levels prevent entry into both S phase and mitosis. (B) Images of *cdk1as/cdk2*$^{-/-}$ cells quantified in Figure 3A´. (C) Images of *cdk1as/cdk2*$^{-/-}$ cells quantified in Figure 3B´. (D) Roscovitine induces premature CENP-A assembly in chicken DT40 cells. Experiment as in Figure 2C´ except that following 1NM-PP1 synchronization and labeling of the nascent CENP-A-SNAP pool, cells were treated with 100 μM Roscovitine and imaged for CENP-A assembly in G2 phase cells.

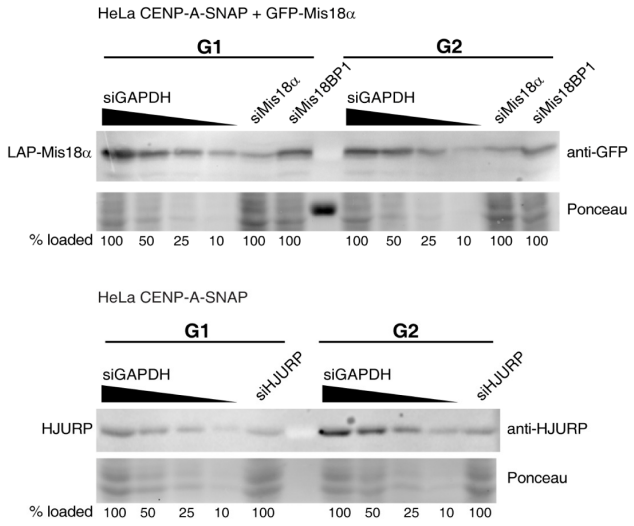

Figure S4. Related to Figure 4. Depletion of Mis18α, Mis18BP1^{HsKNL2} and HJURP by RNAi. HeLa cells expressing CENP-A-SNAP or expressing both CENP-A-SNAP and GFP-Mis18α were transfected with siRNAs, synchronized and drug treated under conditions identical to those in Figure 4E followed by processing for SDS-PAGE and immunoblotting. Fraction of cells loaded is indicated for each condition. Efficiency of depletion of Mis18α and Mis18BP1^{HsKNL2} is assessed by LAP-Mis18α protein levels [Mis18α and Mis18BP1^{HsKNL2} are interdependent (Fujita et al., 2007)] using anti-GFP antibodies. Depletion of HJURP is determined using antibodies against endogenous HJURP.

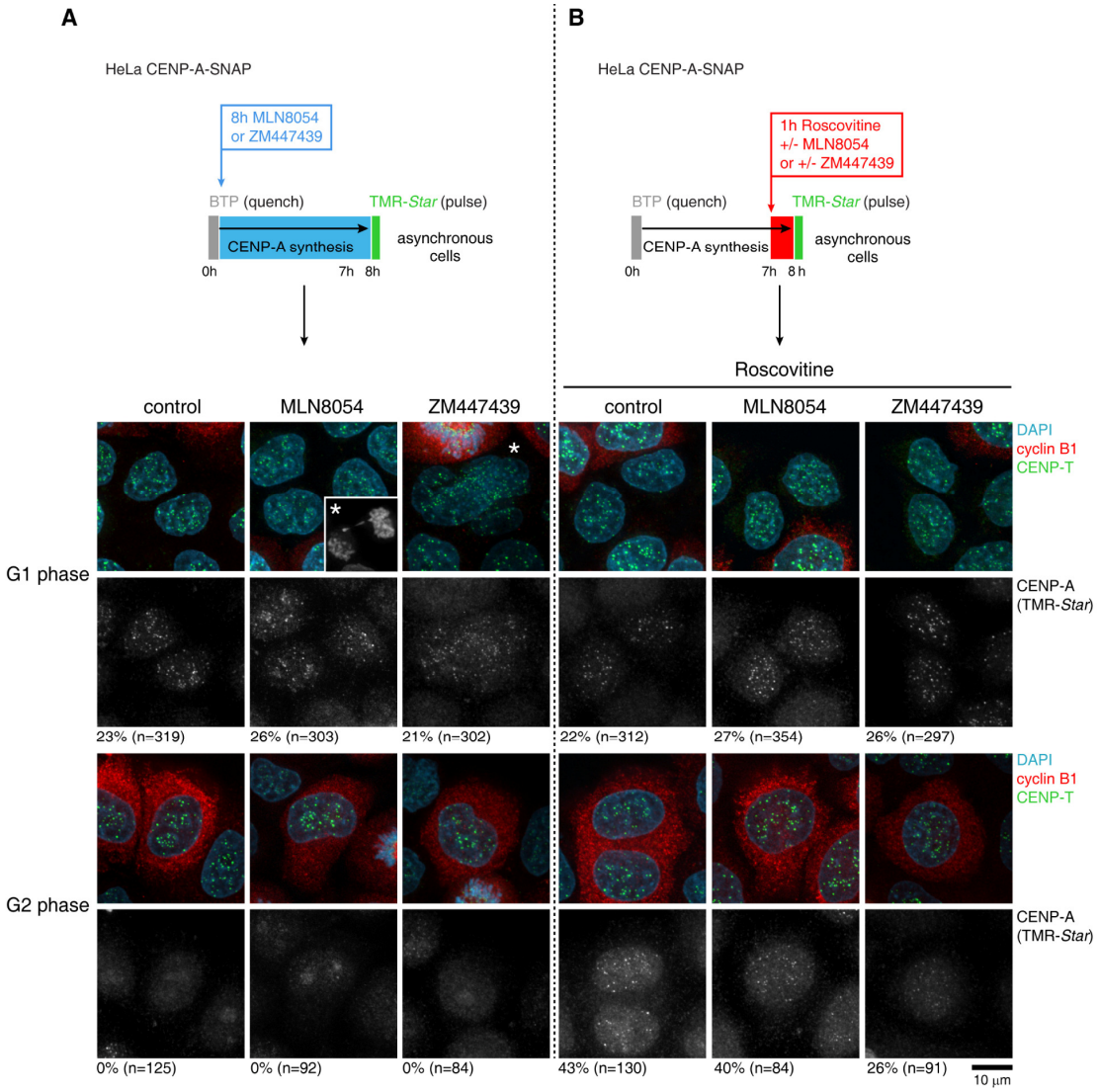

Figure S5. Related to Figure 5. Aurora A and Aurora B activities do not influence the timing of CENP-A assembly. (A) A nascent pool of CENP-A-SNAP was pulse labeled in randomly cycling HeLa cells during which either Aurora A or Aurora B activity was inhibited by treatment with 1µM of MLN8054 or 2µM of ZM447439, respectively. Cells were fixed and counterstained for cyclin B1, CENP-T and with DAPI to indicate G2 status, centromeres and DNA, respectively. Effective kinase inhibition was evident from chromosome segregation defects in mitosis after Aurora A inhibition [indicated by an asterisk and (Hoar et al., 2007)] prior to subsequent CENP-A assembly in G1 or after Aurora B inhibition resulting in cytokinesis failure and multinucleated cells [asterisk and (Ditchfield et al., 2003)]. Representative images of cells in G1 (low cyclin B) and G2 phase (high cyclin B) are shown. (B) Experiment as in A except that cells were treated for one hour with either Roscovitine alone or in combination with MLN8054 or ZM447439 prior to fixation. Percentage of cells assembling CENP-A-SNAP is indicated [either percent of total cells (in top panel, G1 phase) or percent of cyclin B1 positive cells (bottom panel, G2 phase)].

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