REVIEW / SYNTHÈSE

# How to build a centromere: from centromeric and pericentromeric chromatin to kinetochore assembly[1]

**Larissa J. Vos, Jakub K. Famulski, and Gordon K.T. Chan**

**Abstract:** The assembly of the centromere, a specialized region of DNA along with a constitutive protein complex which resides at the primary constriction and is the site of kinetochore formation, has been puzzling biologists for many years. Recent advances in the fields of chromatin, microscopy, and proteomics have shed a new light on this complex and essential process. Here we review recently discovered mechanisms and proteins involved in determining mammalian centromere location and assembly. The centromeric core protein CENP-A, a histone H3 variant, is hypothesized to designate centromere localization by incorporation into centromere-specific nucleosomes and is essential for the formation of a functional kinetochore. It has been found that centromere localization of centromere protein A (CENP-A), and therefore centromere determination, requires proteins involved in histone deacetylation, as well as base excision DNA repair pathways and proteolysis. In addition to the incorporation of CENP-A at the centromere, the formation of heterochromatin through histone methylation and RNA interference is also crucial for centromere formation. The assembly of the centromere and kinetochore is complex and interdependent, involving epigenetics and hierarchical protein–protein interactions.

*Key words:* centromere, kinetochore, pericentromeric chromatin, heterochromatin, RNAi, hierarchiacal assembly.

**Résumé :** L'assemblage du centromère, une région spécialisée de l'ADN associée à un complexe protéique constitutif qui réside à la constriction primaire et qui est le site de formation du kinétochore, a été un casse-tête pour les biologistes pendant de nombreuses années. Des percées récentes dans les domaines de la chromatine, de la microscopie et de la protéomique ont jeté un éclairage nouveau sur ce processus complexe et essentiel. Nous passons ici en revue les découvertes récentes qui concernent les mécanismes et les protéines impliqués dans la détermination de la localisation et de l'assemblage du centromère chez le mammifère. La protéine du cœur du centromère CENP-A, un variant de l'histone H3 essentiel à la formation d'un kinétochore fonctionnel, pourrait, selon les hypothèses, désigner l'endroit où se localise le centromère en s'incorporant dans les nucléosomes spécifiques aux centromères. On a trouvé que la localisation centromérique de CENP-A, et donc la détermination du centromère, requière la présence de protéines impliquées dans la déacétylation des histones ainsi que dans les sentiers de réparation d'ADN par excision de bases et la protéolyse. En plus de l'incorporation de CENP-A dans le centromère, la formation d'hétérochromatine à travers la méthylation des histones et l'interférence à l'ARN est aussi cruciale à la formation du centromère. Les assemblages du centromère et du kinétochore sont complexes et interdépendants, impliquant des phénomènes épigénétiques et des interactions protéine/protéine selon un ordre hiérarchique.

*Mots clés :* centromère, kinétochore, chromatine pericentromérique, hétérochromatine, ARNi, assemblage hiérarchique.

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## Introduction

Mitosis is the process by which a cell equally distributes its chromosomes into 2 daughter cells. The onset of mitosis begins with the breakdown of the nuclear envelope. In prometaphase, the chromosomes attach to the spindle micro-

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tubules through their kinetochores. Once chromosomes are attached to microtubules from opposite spindle poles, it is the balance of forces exerted on the kinetochores that move the chromosomes along the microtubules and eventually aligns them at the spindle equator in metaphase. Once all of the chromosomes are aligned, the cell enters anaphase and the sister chromatids are divided evenly into the daughter cells (for review, see Nigg 2001). How does a cell ensure that, at every mitotic division, each daughter cell gets the correct number of chromosomes? Enter the centromere and kinetochore complexes.

The centromere is a specialized region of chromosomal DNA, along with the constitutive proteins that bind at this site, and can be defined cytologically as the primary constriction visible on condensed chromosomes. It serves as the site of DNA–protein and protein–protein interactions required for the formation of the kinetochore. The proteinaceous kinetochore forms specifically in mitosis and is responsible for microtubule attachment (for review, see Maiato et al. 2004) and the spindle checkpoint (for review, see Chan et al. 2005). The correct segregation of chromosomes is achieved by bipolar attachment of microtubules to the kinetochores on sister chromatids, and fidelity is ensured by the mitotic checkpoint. If there is even a single unaligned chromosome, the mitotic checkpoint will delay anaphase onset until all chromosomes are attached and aligned properly (Rieder et al. 1994). The mitotic checkpoint delays anaphase by inhibiting the anaphase promoting complex/cyclosome, an E3 ubiquitin ligase, and thus prevents the ubiquitination of proteins, such as securin and cyclin B, which need to be degraded before the sister chromatids can separate (King et al. 1995; Sudakin et al. 1995).

Because the centromere and kinetochore protein complexes are involved in such an important cellular process, there are numerous groups looking at how these structures are formed and what the components are. A large number of proteins that localize to the centromeric chromatin are known, and additional members are continuously being identified. Some of these proteins are transiently associated with the centromere, while others are constitutively present and form the structural foundation for all other components. While a small subset of centromere proteins are necessary for centromere formation, they are not sufficient (Fukagawa et al. 1999; Sullivan et al. 1994), and additional mechanisms and (or) components must be required.

In recent years, many reviews have been published on the exciting and complicated structure of the centromere. Here we focus on the components and mechanisms required for centromere formation in vertebrate systems and how the kinetochore assembles upon it. Protein names will be that of the mammalian homolog, except for cases for which research in vertebrate systems is limited. In such cases, data from nonmammalian systems is examined, the mammalian homolog is superscripted, and the organism being discussed is indicated by a prefix. The roles of centromere protein A (CENP-A) and pericentromeric heterochromatin are reviewed in depth because they are both crucial for functional vertebrate centromeres.

## DNA sequence is insufficient for centromere determination

The centromere forms on a specific region of DNA on every chromosome, so it seems logical that the centromere location would be based upon a specific DNA sequence. While this is the case for the simplest of eukaryotes, the budding yeast *Saccharomyces cerevisiae* (sc), it is not so for all other eukaryotes. The centromeric DNA of *Saccharomyces cerevisiae* is a 125 bp sequence that is divided into 3 distinct domains, designated CDEI, CDEII, and CDEIII (Keith and Fitzgerald-Hayes 2000). All other eukaryotes exhibit centromeric DNA that is characterized by highly repetitive tandem sequence repeats (Tyler-Smith and Floridia 2000). A comparison of these repetitive sequences from different species reveals no significant similarity in DNA sequence, and as such, it has been proposed that the centromeric DNA may form conserved structures, such as hairpins, which define the centromere location. To date, 2 centromere-binding proteins, high mobility group A (HMGA) (Disney et al. 1989) and topoisomerase II (Rattner et al. 1996), have been shown to preferentially bind to hairpins, but their roles at the centromere are not well defined (Koch 2000).

Human centromeric DNA ranges in size from less than 200 kb to more than 4 Mb, and is composed of alpha satellite DNA. The human alpha satellite DNA is composed of a core of highly ordered 171 bp repeats, termed α-I satellite DNA, which is framed on either side by divergent repetitive sequences and retrotransposons, referred to as α-II satellite DNA. At the outskirts, the centromeric chromatin becomes rich in long interspersed element 1 (LINE-1 elements) (Schueler et al. 2001). On normal human chromosomes, the centromere forms on a small subdomain of the α-I satellite DNA, but there are cases in which the centromere forms on DNA devoid of α-satellite repeats (du Sart et al. 1997). Studies of abnormal chromosomes, in which the normal centromere is lost or silenced and a new centromere has formed at a new location, have shown that the new site can be completely devoid of α-satellite DNA (du Sart et al. 1997). While these novel centromere locations, called neocentromeres, are generally lacking in α-satellite DNA, they tend to be AT rich and contain LINE elements, 2 characteristics of normal centromeric sites (Warburton 2004). Further evidence that α-satellite DNA doesn't always determine the centromere location comes from analyses of chromosomes that have undergone a Robertsonian translocation. This translocation results in the end-to-end fusion of 2 chromosomes, and as such, there are 2 α-satellite rich centromeric regions. The chromosome remains mitotically stable because only 1 of the centromeres forms a functional kinetochore, even though there are 2 sites of α-satellite DNA (Warburton et al. 1997). While both lines of evidence indicate that α-satellite DNA is not sufficient to dictate centromere location, there are also studies that point to the importance of α-satellite DNA containing a specific sequence, the 17 bp CENP-B-box, for centromere assembly on artificial chromosomes (Ikeno et al. 1998; Ohzeki et al. 2002).

Although centromere formation seems to prefer α-satellite DNA, it is neither required nor sufficient for centromere determination. Since neither the primary DNA sequence nor the presence of α-satellite DNA dictates the centromere location within the large region of DNA, there must be additional factors involved.

## Centromeres are formed by the ordered assembly of proteins

In addition to the DNA component, the centromere also contains numerous proteins, several of which have been identified as constitutive: CENP-A, CENP-B, CENP-C (Earnshaw and Rothfield 1985), CENP-G (He et al. 1998), CENP-H (Fukagawa et al. 2001), CENP-I (Nishihashi et al. 2002) CENP-U(50) (Minoshima et al. 2005), and Mis12 (Goshima et al. 2003) (see Table 1). All of these proteins are essential, except for CENP-B (Kapoor et al. 1998) and CENP-U(50) (Minoshima et al. 2005). hCENP-A, -B, and -C, the first identified centromere proteins, were originally identified as the antigens recognized by antisera from patients with CREST (calcinosis, Raynaud's phenomenon, esophageal dysmotility, sclerodactyly, and telangiectasia) autoimmune disease (Earnshaw and Rothfield 1985). In addition to hCENP-A, -B, and -C, the use of autoimmune patient sera has been a powerful tool in the discovery and study of centromeric and kinetochore proteins, including hCENP-F (an autoimmune disease with assorted characteristics) (Rattner et al. 1993) and hCENP-G (GAVE (gastric antral vascular ectasia) syndrome) (He et al. 1998).

The assembly of these components at the centromere is a sequential and ordered process in which the pathways are branched with interdependency (see Fig. 1*a*). Dependency studies provide information about the order in which the centromere is assembled and functional data for the proteins involved. Through these studies it has been found that, in mammalian systems, there are 2 separate centromere assembly pathways, 1 through CENP-A, and the other through Mis12 (Goshima et al. 2003).

## CENP-A, a core centromere component for kinetochore assembly

To date, the most extensively studied centromere protein is CENP-A. CENP-A is a histone H3 variant that shows 57% homology in the carboxy-terminal histone fold domain, but very little sequence homology in the amino-terminal histone tail. Histone H3 is one of 4 core histone proteins, and it along with histone H2A, H2B, and H4 are incorporated into nucleosomes around which DNA is wrapped to give chromatin its basic packaging subunit. In an in vitro nucleosome reconstitution assay, CENP-A can replace histone H3, and along with equimolar amounts of histone H2A, H2B, and H4, forms a nucleosome with 120–150 bp of DNA wrapped around it (Yoda et al. 2000). Reconstituted CENP-A nucleosomes are of the correct size and molecular weight. The CENP-A nucleosome consists of a complex of 2 CENP-As in a tetramer with histone H4, along with 2 histone H2A/H2B heterodimers (Black et al. 2004). This indicates that, other than the incorporation of CENP-A, the nucleosome retains the same structural components. This is consistent with the immunofluorescence colocalization analysis, which showed that histones H2A and H2B were colocalized with CENP-A, while histone H3 rarely overlapped with CENP-A on metaphase chromosomes. When chromatin fibers are examined by immunofluoresence and fluorescence in situ hybridization, CENP-A and histone H3 are found to be interspersed along the length of the centromeric DNA (Blower et al. 2002) (see Fig. 2*a*). This supports the repeat subunit model of centromere assembly proposed by Zinkowski et al. (1991) to explain the observations that dissociated kinetochores were composed of fragments, each of which retained the ability to bind microtubules, and that, in stretched centromere fibers, the CREST antigens were distributed in a repetitive manner with spacer DNA in between (Zinkowski et al. 1991). There are 2 models that explain how the linear data and 3-D metaphase arrangement can be reconciled. The solenoid model has the chromatin spiraling to form a cylinder, while the other model is a looping model in which the chromatin loops back on itself rather than spiraling. Both models have the end result of separating CENP-A and histone H3 nucleosomes into 2 domains (Blower et al. 2002).

Although the histone H3/H4 and CENP-A / histone H4 tetramers have the same stoichiometry, they have different physical properties. The CENP-A tetramer is more compact than the histone H3/H4 tetramer. This is not due to a size difference, because CENP-A is actually larger than histone H3, albeit only by 4 amino acids. The compact nature of the CENP-A tetramer correlates with the finding that CENP-A is more tightly bound to histone H4, and thus forms a more rigid conformation (Black et al. 2004). The differences seen for the CENP-A / histone H4 tetramer are postulated to provide greater structural integrity to withstand the tension that the centromere is under during bipolar attachment and metaphase alignment.

There are 2 major questions in the field of CENP-A research that still need to be answered. How is CENP-A assembled into nucleosomes and how is the CENP-A nucleosome specifically deposited at the centromere? The majority of studies that look at the incorporation of CENP-A at the centromere have been performed in the fission yeast *Schizosaccharomyces pombe* (sp). Several proteins have been identified which, when mutated, affect CENP-A localization to the centromere.

spMis6$^{CENP-I}$ and spMis12 were identified in a genetic screen for minichromosome instability (Takahashi et al. 1994) and may influence chromosome loss by affecting the ability of spCnp1$^{CENP-A}$ to localize to the centromere. An analysis of spCnp1$^{CENP-A}$ localization revealed that, while spCnp1$^{CENP-A}$ is not altered in spMis12 mutants, the localization of spCnp1$^{CENP-A}$ to the centromere is greatly diminished in spMis6$^{CENP-I}$ mutants. The localization of spMis12 was not affected in spMis6$^{CENP-I}$ mutants and neither spMis6$^{CENP-I}$ nor spMis12 was affected in spCnp1$^{CENP-A}$ mutant cells (Takahashi et al. 2000). spMis6$^{CENP-I}$ interacts with the protein spSim4$^{CENP-H}$ and mutants in spSim4$^{CENP-H}$ also have reduced spCnp1$^{CENP-A}$ localization at the centromere (Pidoux et al. 2003). However, this spCnp1$^{CENP-A}$ recruitment role of spMis6$^{CENP-I}$ and spSim4$^{CENP-H}$ is not conserved. The vertebrate homologs CENP-I and CENP-H, both require CENP-A for centromere localization and neither, when mutated or lost, have an affect on CENP-A localization (Goshima et al. 2003; Nishihashi et al. 2002).

Recently, 4 additional proteins, spMis15–spMis18, were identified as being involved in spCnp1$^{CENP-A}$ recruitment in *Schizosaccharomyces pombe*. spMis15 and spMis17 form a complex with spMis6$^{CENP-I}$, and presumably also with spSim4$^{CENP-H}$, while spMis16 and spMis18 form a separate complex. The spMis16/Mis18 complex is furthest upstream in the spCnp1$^{CENP-A}$ recruitment pathway, as the spMis6$^{CENP-I}$/

**Table 1.** Centromere and kinetochore proteins.

| Vertebrate protein name[a] | Role in mitosis | Depletion or loss-of-function phenotype | Localization dependency | Homologues | References |
|---|---|---|---|---|---|
| **Centromere proteins** | | | | | |
| CENP-A | Histone H3 variant that forms a centromere-specific nucleosome; separate assembly pathway from Mis2, required for localization of all centromere proteins except Mis12 | Kinetochore-null phenotype; embryonic lethal; inability to localize CENP-C, CENP-H, CENP-I, Ndc80 complex, CENP-E and Mad2; BubR1 present but not stable at kinetochore | RbAp46 and RbAp48 in human cells and numerous proteins identified in yeast; see text | Cse4 (sc[b])<br>Cnp1/Sim4 (sp)<br>HCP-3/CENP-A (ce)<br>Cid/CENP-A (Dros) | (Chen et al. 2003; Earnshaw and Rothfield 1985; Goshima et al. 2003; Hayashi et al. 2004; Lehnertz et al. 2003; Regnier et al. 2005; Yoda et al. 2000) |
| CENP-B | Not essential; may assist in centromere site determination | No mitotic defects | 17 bp DNA sequence: CENP-B box | CBF1 (sc)<br>Abp1, Cbh1, Cbh2 (sp) | (Kapoor et al. 1998; Muro et al. 1992; Ohzeki et al. 2002) |
| CENP-C | Specifies localization of KNL proteins Bub1 and BubR1 | Kinetochore-null phenotype; cell death | Requires CENP-A | Mif2 (sc)<br>Cnp3 (sp)<br>HCP-4 /CENP-C (ce) | (Desai et al. 2003; Fukagawa et al. 1999; Goshima et al. 2003; Kalitsis et al. 1998; Oegema et al. 2001) |
| CENP-G | Unknown | Unknown | Unknown | nyi[c] | (Gimelli et al. 2000; He et al. 1998) |
| CENP-H | Specifies localization of Ndc80 complex and CENP-50; required for CENP-C localizationin DT40 chicken cells | CENP-H: inability to localize CENP-C in chicken DT40 cell line; metaphase arrest | Requires CENP-A | CENP-H:Nnf1 (sc)[d]<br>Sim4 (sp) | (Fukagawa et al. 2001; Liu et al. 2003; Mikami et al. 2005; Minoshima et al. 2005; Nishihashi et al. 2002; Sugata et al. 2000) |
| CENP-I | | CENP-I: cell cycle delay in G2; inability to localize Ndc80 complex, CENP-F, Mad1, Mad2 to kinetochores | | CENP-I: Ctf3 (sc)<br>Mis6 (sp) | |
| CENP-U(50) | Unknown | Unknown | Requires CENP-H and CENP-I | nyi | (Minoshima et al. 2005) |
| Mis12 | Separate assembly pathway from CENP-A, required for localization of Ndc80 and PMF1 complexes and Zwint-1 | Mitotic delay due to unaligned chromosomes; inability to localize PMF1, DC8, c20orf172, Zwint-1 | Requires HP1 but not CENP-A | Mtw1 (sc)<br>Mis12 (sp)<br>MIS-12 (ce) | (Goshima et al. 2003; Goshima and Yanagida 2000; Kops et al. 2005; Obuse et al. 2004; Takahashi et al. 1994) |

**Table 1** (*continued*).

| Vertebrate protein name[a] | Role in mitosis | Depletion or loss-of-function phenotype | Localization dependency | Homologues | References |
|---|---|---|---|---|---|
| PARP-1<br>PARP-2 | Unknown; interacts with CENP-A, CENP-B and Bub3 (not CENP-C) and poly(-ADP-ribosyl)ates them following DNA damage | Telomere shortening, chromosome fusion and aneuploidy | Unknown | nyi | (Earle et al. 2000; Menissier de Murcia et al. 2003; Saxena et al. 2002*a*, 2002*b*) |
| **CENP-A-recruiting proteins** | | | | | |
| RbAp46<br>RbAp48 | Histone deacetylation and CENP-A recruitment to centromere | Disrupted CENP-A localization at the centromere | Unknown | Mis16 (sp) | (Hayashi et al. 2004) |
| **Kinetochore proteins** | | | | | |
| PMF1<br>DC8<br>c20orf172 | Involved in chromosome segregation | Mitotic delay due to lagging chromosomes | Requires Mis12 | PMF1: Nnf1 (sc)[d]<br>DC8: Nsl1 (sc)<br>Mis14 (sp)<br>c20orf172: Dsn1 (sc) | (Euskirchen 2002; Obuse et al. 2004; Pinsky et al. 2003) |
| Zwint-1 | Specifies localization of Zw10/Rod/Zwilch complex; essential for mitotic checkpoint | Loss of mitotic checkpoint in the presence of microtubule drugs; inability to localize Zw10, dynamitin, CENP-F and Ndc80 complex to kinetochore | Requires Mis12 and codependent with Ndc80 complex | nyi | (Emanuele et al. 2005; Kops et al. 2005; Obuse et al. 2004; Starr et al. 2000; Wang et al. 2004) |
| Zw10<br>Rod<br>Zwilch | Essential for mitotic checkpoint; recruits Dynein/dynactin and Mad1/Mad2 complexes to kinetochore | Zw10/Rod: no mitotic checkpoint in the presence of microtubule drugs; inability to localize to kinetochore<br>Zwilch: unknown | Zw10 requires Zwint-1; Zw10 and Rod depend on each other | Zw10: ZW-10 (ce)<br>ZW10 (Dros)<br>Rod: F55G1.4 (ce)<br>Rod (Dros) | (Basto et al. 2000; Buffin et al. 2005; Chan et al. 2000; Kops et al. 2005; Scaerou et al. 2001; Starr et al. 1997, 1998; Wang et al. 2004; Williams and Goldberg 1994; Williams et al. 2003) |
| Dynein<br>p150$^{Glued}$ (subunit of Dynactin) | Kinetochore motor; transports Mad2 and Rod poleward after metaphase alignment | Mitotic arrest; inability to remove Mad2 from kinetochores | Requires Zw10/Rod/Zwilch | Dynein: Dyn1 (sc)<br>Dhc1 (sp)<br>Dhc64C (Dros)<br>p150$^{Glued}$: Nip100p (sc)<br>Ssm4p (sp)<br>Glued (Dros) | (Akhmanova and Hoogenraad 2005; Buffin et al. 2005; Harborth et al. 2001; Howell et al. 2001; Starr et al. 1998; Wojcik et al. 2001) |

**Table 1** (*continued*).

| Vertebrate protein name[a] | Role in mitosis | Depletion or loss-of-function phenotype | Localization dependency | Homologues | References |
|---|---|---|---|---|---|
| Ndc80/Hec1<br>Nuf2<br>Spc24<br>Spc25 | Stabilizes kinetochore mictrotubule interaction; recruitment of Mad1 and Mad2; essential for mitotic checkpoint | Inability to form stable kinetochore microtubule attachments and abnormally long spindles; inability to localize Rod, Zw10, dynactin, Mad1, Mad2, Bub1 and Bub3 | Requires Mis12, KNL-1 and CENP-H/CENP-I and codependent with Zwint-1 | Hec1: NDC80 (sc)<br>Ndc80 (sp)<br>NDC-80 (ce)<br>Nuf2: NUF2 (sc)<br>Nuf2 (sp)<br>Him-10 (ce)<br>Spc24: SPC24 (sc)<br>Spc25: SPC25 (sc)<br>KBP-3 (ce) | (Ciferri et al. 2005; DeLuca et al. 2003, 2005; Emanuele et al. 2005; Janke et al. 2001; McCleland et al. 2003, 2004; Wei et al. 2005) |
| KNL-3 | Only identified in *C. elegans*; required for KNL-1 localization | Inability of KNL-1 and Mis12 to localize to the kinetochore | Codependent with Mis12 | KNL-3 (ce) | (Cheeseman et al. 2004) |
| KNL-1 | Also called AF15q14; affects localization of Ndc80 complex and Bub1 | Kinetochore-null phenotype; loss of Ndc80 complex | Requires Mis12 and KNL-3 | Spc105 (sc)<br>KNL-1 (ce) | (Cheeseman et al. 2004; Desai et al. 2003) |
| CENP-E | Kinetochore kinesin motor required for reliable bioriented spindle microtubule-kinetochore attachment; interacts with and activates BubR1; part of the BubR1/CENP-E mitotic checkpoint mechanosensory complex | Delayed mitosis due to unaligned chromosomes; reduced hyperphosphorylation of BubR1 | Required Bub1 and BubR1 | CENP-meta (Dros) | (Chan et al. 1998, 1999; Kapoor et al. 2006; Mao et al. 2003, 2005; McEwen et al. 2001; Putkey et al. 2002; Wood et al. 1997; Yao et al. 2000; Yen et al. 1991) |
| CENP-F | Stabalizes kinetochore microtubule interactions | Decreased stability of kinetochore microtubule interactions | Requires Zwint-1 and Bub1 | Okp1, Slk19 (sc)[e]<br>HCP-1/HCP-2 (ce) | (Bomont et al. 2005; Cheeseman et al. 2005; Holt et al. 2005; Johnson et al. 2004; Rattner et al. 1993; Wang et al. 2004) |
| Bub1 | Mitotic checkpoint kinase, essential for mitotic checkpoint | No mitotic checkpoint in the presence of microtubule drugs; inability to localize CENP-F, CENP-E, BubR1, Bub3, and Mad2 | Requires KNL-1 and Ndc80 complex | BUB1 (sc)<br>Bub1 (sp)<br>BUB1 (ce) | (Basu et al. 1998; Desai et al. 2003; Jablonski et al. 1998; Johnson et al. 2004; Sharp-Baker and Chen 2001; Taylor et al. 1998) |
| Bub3 | Binds to Bub1 and BubR1; part of mitotic checkpoint complex | No mitotic checkpoint in the presence of microtubule drugs; | Requires Bub1 | BUB3 (sc)<br>Bub3 (sp)<br>BUB3 (ce) | (Basu et al. 1998; Sharp-Baker and Chen 2001; Sudakin et al. 2001; Taylor et al. 1998) |

**Table 1** (*concluded*).

| Vertebrate protein name[a] | Role in mitosis | Depletion or loss-of-function phenotype | Localization dependency | Homologues | References |
|---|---|---|---|---|---|
| BubR1 | Mitotic checkpoint kinase; part of mitotic checkpoint complex | No mitotic checkpoint in the presence of microtubule drugs; required for stable attachment of chromosomes to spindle microtubules | Requires CENP-C and Bub1 | MAD3 (sc)<br>Mad3 (sp)<br>SAN-1/ Mad3 (ce)<br>BubR1 (Dros) | (Chan et al. 1999; Chen 2002; Harris et al. 2005; Jablonski et al. 1998; Johnson et al. 2004; Lampson and Kapoor 2005; Mao et al. 2003, 2005; Regnier et al. 2005; Sudakin et al. 2001) |
| Mad1 | Required for localization of Mad2 | No mitotic checkpoint in the presence of microtubule drugs | Requires Zw10/Rod/Zwilch, Bub1 and BubR1 | MAD1 (sc)<br>Mad1 (sp)<br>MDF-1 (ce) | (Chen et al. 1998; De Antoni et al. 2005; DeLuca et al. 2003; Liu et al. 2003) |
| Mad2 | Part of the mitotic checkpoint complex; binds Cdc20 and inhibits APC/C | No mitotic checkpoint in the presence of microtubule drugs | Requires Mad1, Zw10/Rod/Zwilch, Bub1 and BubR1 | MAD2 (sc)<br>Mad2 (sp)<br>MDF-2 (ce) | (Buffin et al. 2005; Chen et al. 1998; De Antoni et al. 2005; DeLuca et al. 2003; Johnson et al. 2004; Liu et al. 2003; Sudakin et al. 2001) |

[a]Proteins that are either found as or act as a complex have been grouped together, with each individual protein appearing on a separate line.
[b]sc, *Saccharomyces cerevisiae*; sp, *Schizosaccharomyces pombe*; ce, *Caenorhabditis elegans*; Dros, *Drosophila.*
[c]nyi, not yet identified.
[d]Nnf1(sc) shows homology to both human CENP-H and PMF1.
[e]Both Okp1(sc) and Slk19(sc) have limited sequence homology to CENP-F.

Mis15/Mis17 complex and spCnp1$^{CENP-A}$ both required the spMis16/Mis18 complex for localization (Hayashi et al. 2004). Unlike in spMis6$^{CENP-I}$, the function of spMis16 in spCnp1$^{CENP-A}$ localization appears to be conserved in vertebrates. siRNA knockdown of both of the spMis16 human homologs hRbAp46 and hRbAp48 disrupted hCENP-A localization. Analysis of the histone modifications in the *Schizosaccharomyces pombe* mutants or the siRNA treated human cells revealed an increase in the acetylation of both histones H3 and H4 in the centromere domain (Hayashi et al. 2004). Interestingly, hRbAp46 and hRbAp48 are components of a histone deacetylase (HDAC) complex (Zhang et al. 1999), implying that hCENP-A localization depends on hypoacetylated histones at the centromere. However, this is inconsistent with studies that have shown that treatment of human cells with HDAC inhibitors does not affect hCENP-A localization (Robbins et al. 2005). Perhaps hRbAp46 and hRbAp48 have additional functions that contribute to hCENP-A recruitment.

The GATA-transcription factor spAms2 has also been found to play a role in localization of spCnp1$^{CENP-A}$ in *Schizosaccharomyces pombe*. spAms2 was identified in a genetic screen for multicopy suppressors of a spCnp1$^{CENP-A}$ mutant. Although deletion of the spAms2 gene resulted in diminished spCnp1$^{CENP-A}$ nucleosome incorporation at the centromere (Chen et al. 2003) just as seen in the spMis6-$^{CENP-I}$ mutant (Takahashi et al. 2000), the spAms2 protein has several distinct characteristics. The expression of spAms2 is cell cycle regulated, with the highest level in S phase and very little being present in mitosis. As such, it is not a constitutive centromere protein, while the spMis proteins mentioned above are. Another unique characteristic is that spAms2 is localized at sites in the chromatin other than the centromere, presumably at the promoters of GATA-factor responsive genes. Additionally, the loss of spAms2 is not lethal (Chen et al. 2003). These results may be explained by the spAms2 GATA factor being involved in spCnp1$^{CENP-A}$ recruitment indirectly by transcription of a spCnp1$^{CENP-A}$ loading factor, and there may be redundancy because many promoters have binding sites for more than 1 transcription factor. The other possibility is that there may be 2 spCnp1$^{CENP-A}$ recruitment pathways. Several studies have looked at the timing of centromeric DNA replication, and while there is variation among species, there is a general trend of mid-S phase. In cells in which DNA replication was blocked, newly synthesized CENP-A was still able to localize to the centromere, implying a DNA replication independent loading mechanism (Shelby et al. 2000). Perhaps spAms2 participates in the loading of spCnp1$^{CENP-A}$ during S phase and additional factors, such as spMis6$^{CENP-I}$, are able to compensate by recruiting spCnp1$^{CENP-A}$ during G2. GATA factors are conserved, but it remains to be seen whether there is a conserved mechanism in vertebrates.

A recent study in *Xenopus laevis* (xl) egg extracts has pointed to a potential role for base excision repair proteins in xlCENP-A recruitment. Following fertilization, parental xlCENP-A is dissociated from the sperm chromatin and is replaced with maternal xlCENP-A. Examining this process

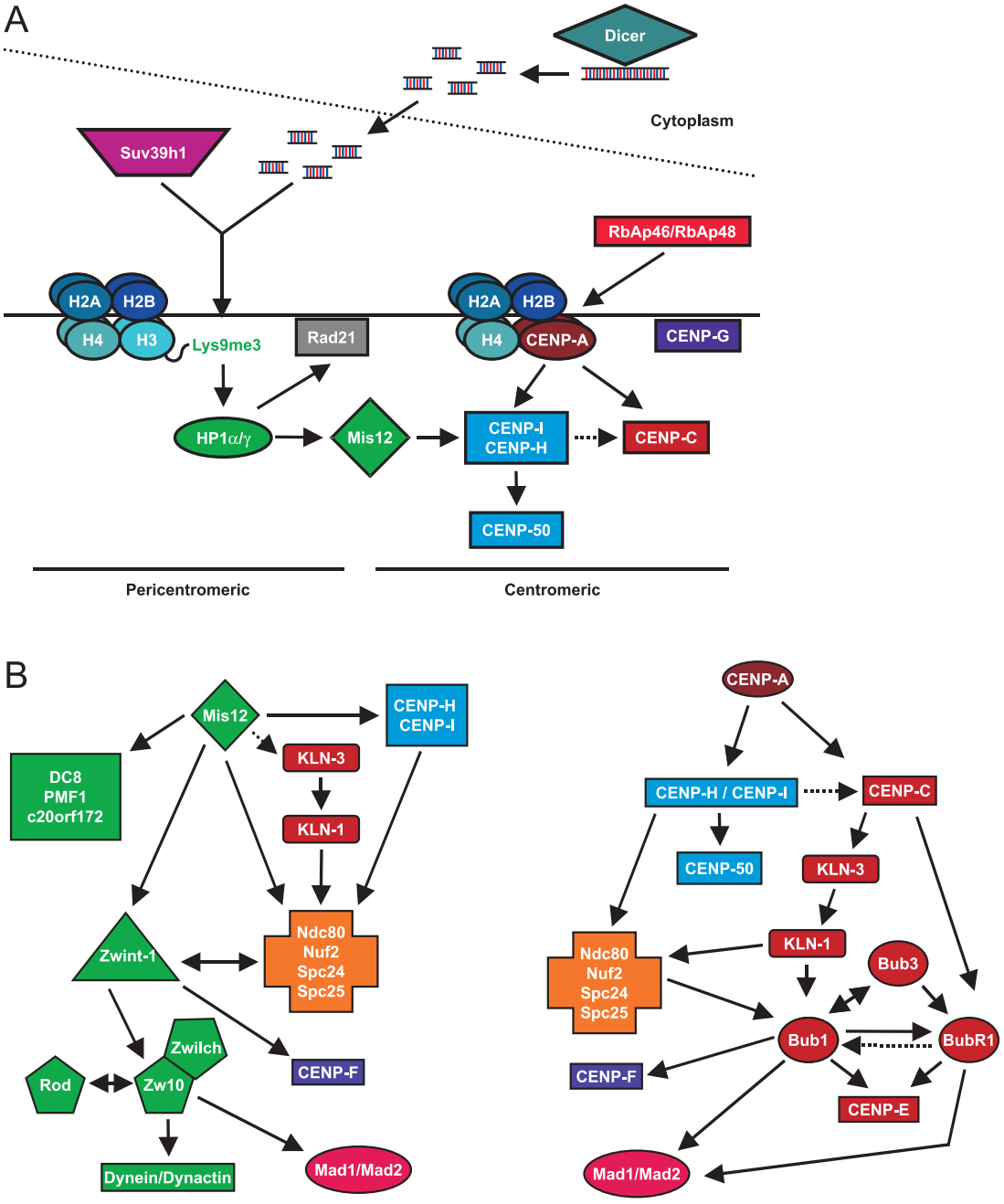
**Fig. 1.** The Hierarchical assembly of the vertebrate centromere and kinetochore. (A) Two centromere pathways: CENP-A and Mis12 are both required for centromere assembly. The centromeric and pericentromeric chromatin form the foundation for centromere assembly. siRNAs, produced by Dicer in the cytoplasm, cooperate with the methyltransferase Suv39h1 to trimethylate histone H3 at lysine 9 in the pericentromeric region. HP1 binds preferentially to this modification and recruits Rad21, a cohesion subunit, as well as Mis12. In the centromeric chromatin, CENP-A replaces histone H3 in the nucleosomes and it is required for the recruitment of CENP-C and the CENP-H/CENP-I complex. The CENP-H/CENP-I complex also requires Mis12 for assembly, and in turn it recruits CENP-50. It is not known how CENP-G localizes to the centromere, and as such, no dependency is indicated. CENP-B is not included because its absence does not affect mitosis in vertebrate systems. Please see text for a detailed discussion of the assembly of CENP-A. Solid arrows represent localization dependency (from numerous organisms) and do not imply direct interaction. Dashed arrows represent localization requirements that are not conserved among vertebrates (required in at least 1 organism but not in others). (B) Kinetochore assembly pathways through Mis12 and CENP-A. Left panel: the Mis12 kinetochore assembly pathway. Right panel: CENP-A kinetochore assembly pathway. The assembly of the kinetochore upon the centromere is complex. While some proteins seem to be exclusively recruited by only a single protein (i.e., in human cells, CENP-C requires only CENP-A for localization and is a member of only the CENP-A pathway), many proteins require more than 1 protein for localization and are bridging proteins between the 2 pathways (i.e., the Ndc80 complex requires Mis12, Zwint-1, KNL-1, and the CENP-H/CENP-I complex). There are also proteins that localize interpedently (i.e., Zw10 and Rod require each other for localization) (see Table 1 for a detailed description of proteins). Protein complexes are indicated by a single shape (e.g., PMF1, DC8, and c20orf172 are members of a complex that interacts and requires Mis12). The color of the shapes represents recruitment pathways. The Mis12 pathway is in green, the CENP-A pathway is in red, and proteins that are involved in both pathways are unique colors (i.e., the Ndc80 complex is a branch point between pathways from CENP-A through the CENP-H/CENP-I complex, and the Mis12 pathway therefore is a distinct color (orange)). Arrows represent dependency and not interaction, as described above.

in *Xenopus* egg extracts identified xlUNG2, a uracil DNA glycosylase, and an unidentified deoxycytidine deaminase as potential determinants for xlCENP-A localization (Zeitlin et al. 2005). When DNA damage was induced by ultraviolet light, ionizing radiation, or chemicals, the size of xlCENP-A foci increased. The number of foci also increased and was greater than that expected, based on the number of centromeres. Perhaps xlCENP-A plays a role in DNA repair, or perhaps additional centromere-like foci are forming in response to DNA damage to increase the probability that a functional centromere will form after the damage is repaired. The *Xenopus* egg extracts used in this study do not proceed through mitosis, and as such, it is not possible to analyze the role that xlUNG2 and the deoxycytidine deaminase play in mitosis. The connection between base excision repair and xlCENP-A loading may be specific for depositing maternal xlCENP-A onto the sperm chromatin following fertilization (Zeitlin et al. 2005). This mechanism may be conserved, at least in embryogenesis, because 2 polyADP-ribosylases, PARP-1 and PARP-2, have been identified in a mouse embryonic stem cell line, which are involved in the base excision repair pathway and have been found to interact with CENP-A. These proteins polyADP-ribosylate CENP-A in response to ionizing radiation (Saxena et al. 2002*a*, 2002*b*), but whether they influence its incorporation remains to be determined.

The centromere-specific localization of CENP-A nucleosomes can be achieved by either specific recruitment to the centromere, selective removal of noncentromeric CENP-A nucleosomes, or a combination of both. In *Saccharomyces cerevisiae*, it appears that both of these mechanisms are responsible. Under normal circumstances, scCse4p$^{CENP-A}$ (the budding yeast CENP-A homolog) is recruited to the centromere by the spCBF3 protein complex (discussed further, below). Unlike overexpression of CENP-A in humans (Van Hooser et al. 2001), overexpression of scCse4p$^{CENP-A}$ showed no mislocalization to euchromatin and no observable phenotype of any kind. Furthermore, there is no increase in cytoplasmic protein, indicating that its level might be regulated by protein degradation (Collins et al. 2004). When a partially stabilized Myc-tagged scCse4p$^{CENP-A}$ (all 16 lysine residues had been mutated to arginine) was overexpressed, mislocalization to euchromatin was observed. This indicated that proteolysis usually prevents Cse4p from localizing to euchromatin. When scCse4p$^{CENP-A}$ was targeted to degradation with a degron sequence, it was found that centromeric scCse4p$^{CENP-A}$ was protected against proteolysis (Collins et al. 2004). It is not clear whether proteolysis plays a role in restricting CENP-A nucleosomes to the centromere in other organisms. In humans, this does not appear to be the case because CENP-A can be overexpressed, although this is not conclusive because it is possible that the proteolysis machinery was overloaded.

A model has been proposed in which kinetochore functions, microtubule attachment, and chromosome segregation mark the centromere location for the next cell cycle (Mellone and Allshire 2003). It is not known whether microtubule attachment or tension across the sister chromatids could produce an epigenetic modification or other type of mark that would specify the location of CENP-A incorporation in the following cycle. In this model, any protein that disrupts kinetochore function would result in the loss of CENP-A recruitment. However, this does not appear to be the case because there are numerous proteins which, when mutated or lost, disrupt kinetochore functions and yet have no effect on CENP-A localization (e.g., Mis12; Goshima et al. 2003). Furthermore, the formation of neocentromeres occurs without a previous mitosis to mark the centromere location, and CENP-A is recruited efficiently (Warburton 2004). There are also cases in which CENP-A, when overexpressed, can load onto the chromatin without forming a functional kinetochore (Van Hooser et al. 2001).

The presence of CENP-A at the centromere is essential for mitosis to occur properly and determines the localization of CENP-C, CENP-H, and CENP-I. The importance of CENP-A is seen in the fact that mice null for CENP-A exhibit an early embryonic lethal phenotype with a high degree of chromosome missegregation (Howman et al. 2000).



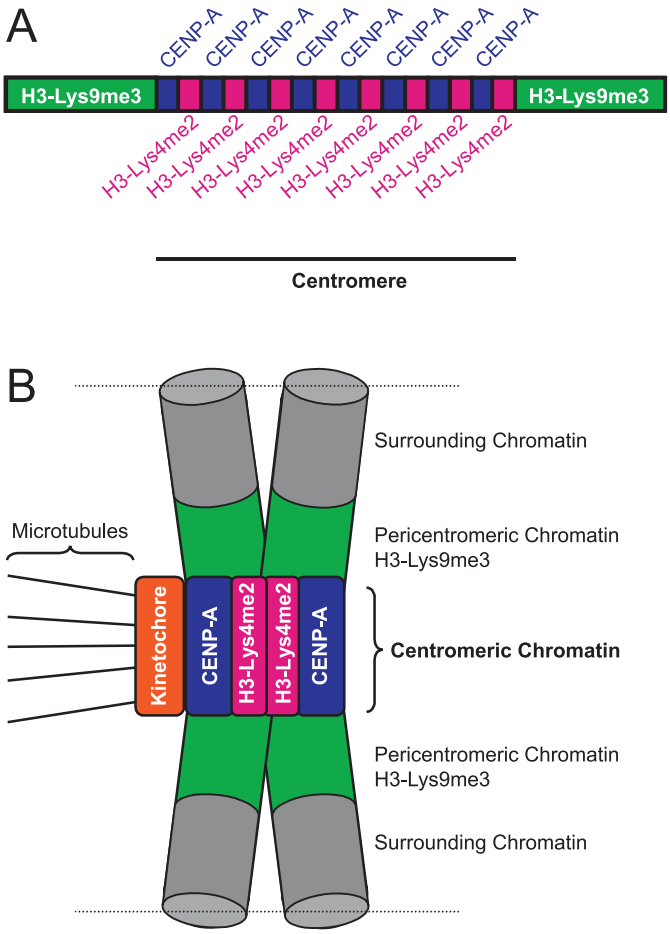

These observations lead to the hypothesis that CENP-A determines the location of the centromere. Studies in which CENP-A is overexpressed have shown that, although CENP-A is necessary, it is not sufficient for kinetochore formation (Van Hooser et al. 2001). When CENP-A is overexpressed, its localization is no longer restricted to centromeres and it can be seen at random locations along the chromosome arms. CENP-C, hZwint-1 (see Table 1), and hSMC1, a cohesin subunit, were found to localize to these noncentromere sites, but these sites are not functional as centromeres, in that they cannot specify kinetochore assembly (Van Hooser et al. 2001). This indicates that there must be additional properties of the centromere, other than the CENP-A-containing nucleosome, which are required for functional kinetochore formation.

## Epigenetics of the centromeric and pericentromeric chromatin determine centromere location

The structure and function of chromatin is regulated by epigenetic modifications. Epigenetic modifications are changes to the genome that do not involve altering the DNA sequence and are heritable through mitosis and meiosis. There are 3 main types of epigenetic mechanisms: DNA methylation, gene imprinting, and histone modifications (for review, see Nakao 2001).

DNA methylation is the covalent attachment of a methyl group to carbon 5 of the cytosine ring and is catalyzed by a group of enzymes called DNA methyl-transferases. This modification is reversible by demethylating enzymes, in-

**Fig. 2.** The Centromeric chromatin contains a unique epigenetic modification. (A) Schematic of a stretched chromatin fiber. Centromeric chromatin consists of CENP-A nucleosomes interspersed with histone H3 nucleosomes. The histone H3 nucleosomes in the centromeric region have a unique epigenetic modification, dimethylation of histone H3-Lys4 (H3-Lys4me2), which is different from that of the surrounding pericentromeric heterochromatin. (B) Distinct 3-D domains of histone H3 and CENP-A containing nucleosomes. In metaphase chromosomes, the histone H3-Lys4me2 is located at the site of sister chromatid cohesion and CENP-A at the exterior surface facing toward the spindle poles. The centromeric chromatin is surrounded on either side by trimethylated histone H3-Lys9 (H3-Lys9me3) nucleosomes at the pericentromeric heterochromatin. This positioning of the CENP-A domain might specify the assembly of the kinetochore in the proper orientation for microtubule attachment.




cluding 5-methylcytosine glycosylase (Fremont et al. 1997). These enzymes are tightly regulated because DNA methylation plays a key role in the regulation of gene expression. Gene imprinting is the silencing of 1 parental allele, while the other allele remains actively expressed. DNA methylation is required to maintain this allele-specific silencing (Li et al. 1993).

The epigenetic modifications that can occur on the amino-terminal tails of histones include methylation, acetylation, and phosphorylation (for review, see Dunleavy et al. 2005). These modifications can either generate or remove binding sites for chromatin-associated proteins which, in turn, affect gene expression through chromatin structure. Because more than a single modification can occur on each histone tail, the histone code provides an exponential increase in the information that can be encoded, compared with the genetic code (Jenuwein and Allis 2001). Transcriptionally inactive regions, termed heterochromatin, tend to be hypoacetylated on histones H3 and H4. Additionally, methylation of histone H3 on lysine 9 (H3-Lys9) is correlated with gene silencing and heterochromatic DNA (Bannister et al. 2001). In contrast, histone H3-Lys9 is acetylated and histone H3-Lys4 is methylated in transcriptionally active euchromatin (Santos-Rosa et al. 2002). Acetylation of histone tails is regulated by histone acetyltransferases and HDACs.

CENP-A differs from histone H3 mostly in the amino-terminal tail, where epigenetic modifications occur. Because of the prominent role of histone epigenetic modifications, the potential of the amino-terminal tail of CENP-A to be epigenetically modified has been examined. The only residues that are conserved between hCENP-A and histone H3 is an arginine residue at position 8 and a lysine at position 9 (Van Hooser et al. 1999). In histone H3, this Lys9 can be either acetylated or methylated, but no epigenetic modifications have been found at this residue in hCENP-A. The only epigenetic modification of hCENP-A that has been identified to date is the phosphorylation of serine at position 7 by Aurora B kinase (Zeitlin et al. 2001). This serine at position 7 is not conserved between the CENP-A homologs, being found only in humans and not mouse, goat, or bovine. As such, it is doubtful that phosphorylation of serine 7 has a regulatory function. At this time, it is not clear if epigenetic modifications regulate the CENP-A nucleosome structure.

The centromere is transcriptionally silent and bordered on each side with heterochromatin (Sullivan et al. 2001). Thus, it is expected that the centromere will contain heterochromatic epigenetic modifications, specifically dimethylated or trimethylated histone H3-Lys9 (H3-Lys9me2/me3) and hypoacetylation of both histones H3 and H4. When extended centromeric fibers were analyzed, no methylated histone H3-Lys9 was detected in the centromeric region, although it was found in the heterochromatin on either side of CENP-A containing chromatin. By analyzing other histone modifications, it was found that histone H3-Lys4 was dimethylated (H3-Lys4me2) in the centromeric chromatin domain (Sullivan and Karpen 2004) (see Fig. 2*a*), which was unexpected because dimethylation of histone H3-Lys4 is a marker typically associated with euchromatin. However, other epigenetic modifications of euchromatin, such as hyperacetylation of histones H3 and H4, were not present (Jeppesen et al. 1992). Interestingly, the heterochromatic modification of histone H3 Ser10 phosphorylation (H3-Ser10ph) (Hendzel et al. 1997) was also absent from the centromeric chromatin domain. Thus, the epigenetic modifications of the centromeric chromatin are distinct from that of the surrounding heterochromatin and also from euchromatin. This unique epigenetic modification combination may be how the centromere location is determined. In metaphase chromosomes, the dimethylated histone H3-Lys4 was found on the internal face of the chromosome, where sister chromatid cohesion occurs, while the CENP-A signal was on the outside face where the kinetochore is located, facing the spindle pole (Sullivan and Karpen 2004). This

positioning of the CENP-A domain might specify the assembly of the kinetochore in the proper orientation for microtubule attachment (see Fig. 2*b*).

The centromeric chromatin exists within pericentromeric heterochromatin, which is required for proper kinetochore assembly. The epigenetic modification that is essential for vertebrate heterochromatin formation is trimethylation of histone H3 at lysine 9 (H3-Lys9me3). This methylation is catalyzed by the methyltransferase enzyme Suv39h1 (Rea et al. 2000), and creates a binding site for the heterochromatin protein HP1 (Lachner et al. 2001). HP1 in turn recruits the DNA methyltransferase 3b, leading to silenced pericentromeric heterochromatin. Another function of HP1 is the recruitment of the cohesin subunit Rad21, required for sister chromatid cohesion (Nonaka et al. 2002). Both Suv39h1 and HP1 contain a chromodomain, a motif that can bind both DNA and RNA, and are evolutionarily conserved from *Schizosaccharomyces pombe* through to humans (Eissenberg and Elgin 2000). It is the ability of the chromodomain of each protein to interact with DNA and RNA that is crucial for its function in centromere silencing and accurate chromosome segregation (Allshire et al. 1995; Nakayama et al. 2000). Loss of Suv39h1 or HP1 results in aberrant chromosome segregation, premature sister chromatid separation, aneuploidy, and loss of silencing at the pericentromeric region (Guenatri et al. 2004; Lehnertz et al. 2003; Rea et al. 2000; Taddei et al. 2001).

Mammals have 3 isoforms of HP1 (HP1α, HP1β/M31, and HP1γ/M32), and all 3 interact specifically with histone H3 (Nielsen et al. 2001). As the name implies, all 3 HP1 isoforms localize to heterochromatin at the centromere, but HP1γ is also able to bind to euchromatin (Minc et al. 2000), although how HP1γ associates with euchromatin is not known. The chromodomain of HP1 proteins binds to histone H3 trimethylated at lysine 9 (H3-K9me3) (Bannister et al. 2001; Lachner et al. 2001), but this modification is not present in euchromatin. The epigenetic state of histone H3 in euchromatin is acetylation of lysine 9 (H3-Lys9ac) and dimethylation of lysine 4 (H3-Lys4me2) (Cheung and Lau 2005). Studies have found that HP1 proteins are not able to associate with histone H3-Lys4me2 (Bannister et al. 2001; Jacobs et al. 2001) and that hyperacetylation diminishes HP1 binding (Robbins et al. 2005; Taddei et al. 2001). As such, HP1γ must interact with a euchromatin binding protein, but the identity of this protein is not known.

In addition to epigenetic modifications, another mechanism involved in determining chromatin structure is the incorporation of histone variants. The histone H2A variant H2A.Z is found exclusively in heterochromatin and is essential in both *Drosophila* (van Daal and Elgin 1992) and mice (Faast et al. 2001). Several studies have examined the role of H2A.Z and have found that it is localized at pericentromeric heterochromatin and interacts with HP1α (Fan et al. 2004). When histone H2A.Z is knocked down by RNA interference (RNAi), HP1α localization is lost from heterochromatin, including pericentromeric heterochromatin, but HP1α remains at the centromere (Rangasamy et al. 2004). It has also been found that the interaction of HP1α with heterochromatin depends upon the N-terminal tail of histone H4 (Fan et al. 2004). This indicates that, while HP1 may be able to specifically interact with histone H3 trimethylated at lysine 9, there are additional factors that affect its interaction with heterochromatin.

Despite the observed interaction and requirement of H2A.Z and H4 for HP1 localization to heterochromatin, the current model for heterochromatin formation is that Suv39h1 is recruited to, and trimethylates, histone H3-Lys9, which in turn binds HP1. This model may be correct for heterochromatin at all sites except for the centromere, where it has been shown that these proteins are dynamic and have changes in localization during the cell cycle. In interphase cells, Suv39h1 and HP1 proteins colocalize at pericentromeric heterochromatin and exhibit no colocalization with CENP-A (Aagaard et al. 1999), but in mitotic cells, Suv39h1 and HP1 occupy distinct domains. Suv39h1 dissociates from the pericentromeric heterochromatin and localizes to the kinetochores during prometaphase and metaphase. At the metaphase-to-anaphase transition, it dissociates from the kinetochore. The change in Suv39h1 location corresponds to the time when phosphorylated species of Suv39h1 become apparent in extracts (Aagaard et al. 2000). It is not known whether phosphorylation of Suv39h1 plays a regulatory role in Suv39h1 kinetochore association. It is also not clear if the role of the mitotic Suv39h1 at kinetochores is to maintain and (or) alter the methylation status of the centromeric chromatin.

HP1 proteins also have an altered localization in mitosis. At the onset of mitosis, the majority of HP1 protein dissociates from the pericentromeric heterochromatin (Minc et al. 1999; Sugimoto et al. 2001). A small population of HP1α remains bound at the centromeric chromatin boundary and shows partial colocalization with CENP-A (Sugimoto et al. 2001). The histone H3-Lys9me3 epigenetic mark remains at the pericentromeric heterochromatin (Fischle et al. 2005; Hirota et al. 2005), so it is not the redistribution of Suv39h1 which causes the dissociation of HP1 in this region. At mitosis, there is a cell-cycle-specific epigenetic modification of histone H3-Ser10 phosphorylation (H3-Ser10ph) by the Aurora B kinase (Adams et al. 2001; Hendzel et al. 1997; Hsu et al. 2000). The phosphorylation of serine 10 occurs on the same histone H3 as the trimethylation of lysine 9, and in vitro binding assays show that HP1 has a reduced affinity for this doubly modified histone. The inhibition of Aurora B results in the loss of histone H3-Ser10ph and retention of HP1 binding at pericentromeric heterochromatin (Fischle et al. 2005; Hirota et al. 2005). Future studies will determine what role this dynamic HP1 has on mitosis.

## siRNAs guide histone methyltransferases and heterochromatin formation

In addition to epigenetic modifications, recent studies have found that RNAi plays a role in establishing heterochromatin and affects chromosome segregation. RNAi was originally identified in the nematode worm *Caenorhabditis elegans* (Fire et al. 1998) and in plants (Hamilton and Baulcombe 1999). It is conserved from fission yeast up to humans, including in plants. The several possible outcomes of RNAi include degradation of mRNA, inhibition of transcription, changes in chromatin structure, or a combination of the 3 (Bernstein and Allis 2005). An RNA endonuclease, Dicer, cleaves long double-stranded RNA (dsRNA) or hairpin RNA

(hRNA) into 20–25 nucleotide fragments, termed small interfering RNA (siRNA), which become incorporated into the RNA-induced silencing complex (RISC). The RISC complex targets the homologous mRNA, and the RNA-induced initiation of transcriptional silencing complex targets DNA for silencing (for review, see Hannon 2002).

The majority of studies on the roles of RNAi in cellular processes have been done in *Schizosaccharomyces pombe* because it has a single homolog of each of the RISC components Dicer, Argonaute, and RNA-dependent RNA polymerase (Rdp1) (Volpe et al. 2002), making it easy to delete each component without the worry of compensation or rescue by a paralog. Deletion of any one of these 3 RISC components leads to the loss of silencing of reporter genes inserted into the pericentromeric heterochromatin (Provost et al. 2002; Volpe et al. 2002). The loss of RNAi function also results in the loss of histone H3-Lys9 methylation, dissociation of the *Schizosaccharomyces pombe* HP1 homolog spSwi6$^{HP1}$ (Volpe et al. 2002), and mitotic defects (Hall et al. 2003; Provost et al. 2002; Volpe et al. 2002). An abnormal accumulation of pericentromeric repeat transcripts was found in *Schizosaccharomyces pombe* RISC mutants, pointing to the critical role of Dicer in degrading these transcripts (Volpe et al. 2002). Because both the fission yeast methyltransferase spClr4$^{Suv39h1}$ and spSwi6$^{HP1}$ contain chromodomains (Akhtar et al. 2000; Eissenberg and Elgin 2000), and this motif has RNA binding properties, it is possible that the siRNAs produced by Dicer are used to target these 2 proteins to the heterochromatin, where they participate in the silencing.

Further evidence that the RNAi machinery plays a role in determining heterochromatin comes from the expression of a synthetic dsRNA hairpin homologous to the *ura4*$^+$ gene (Schramke and Allshire 2003). This gene is normally constitutively expressed, but the introduction of the dsRNA hairpin causes silencing and heterochromatin formation. The region becomes marked with methylated histone H3-Lys9, binding of spSwi6$^{HP1}$, and recruitment of spRad21 (cohesin subunit), characteristics of the pericentromeric heterochromatin. The methyltransferase spClr4$^{Suv39h1}$ is required for this RNAi silencing because siRNAs are not produced from the dsRNA hairpin in spClr4$^{Suv39h1}\Delta$ cells (Schramke and Allshire 2003).

The role of siRNA in heterochromatin production is difficult to study in vertebrate systems. The Dicer knockout mouse is not viable because of an embryonic lethal phenotype (Bernstein et al. 2003). As such, conditional knockout systems are required. Using a chicken–human hybrid cell line derived from DT40, with the expression of Dicer under a tetracycline repressible promoter, it has been shown that RNAi is also needed for heterochromatin formation in vertebrate cells (Fukagawa 2004). The loss of Dicer expression results in the accumulation of transcripts homologous to the centromere of human chromosome 21 and dissociation of HP1 and Rad21 (cohesin) from pericentromeric heterochromatin. Before the cells die, they accumulate in mitosis and exhibit premature sister chromatid separation. Analysis revealed that CENP-A and CENP-C localization to centromeres are not affected in Dicer$^{-/-}$ cells. Although there is an accumulation of mitotic cells, there is no mitotic block, and the mitotic kinase BubR1 (see Table 1) is absent from prematurely separated sister chromatids, indicating that there is a loss of the mitotic checkpoint (Fukagawa et al. 2004). The role of RNAi in heterochromatin assembly is conserved and explains the previously confounding result that treatment of mouse cells with RNase caused release of HP1 from pericentromeric heterochromatin (Maison et al. 2002). Future studies will determine how RNAi modifies chromatin in higher eukaryotes.

## The assembly of the centromere and kinetochore proteins complexes is complex and hierarchical

Numerous studies from many different organisms have begun to elucidate how the kinetochore forms on the centromere at mitosis. As with the CENP-A recruitment studies, there are contradictory results on how the kinetochore assembles. This indicates that, while the function and components of the kinetochore may be conserved, there are a variety of ways to build a kinetochore.

Studying the kinetochore has proven to be challenging in vertebrate systems because it is an insoluble complex formed only in mitosis and some proteins have low abundance or cell-cycle regulated expression. Initial studies of kinetochore proteins were performed in budding and fission yeast. These small eukaryotes can be grown to large numbers for the isolation of low-abundance proteins. In addition, genetic screens can be performed to analyze mutants with chromosome segregation defects. Through these genetic screens, the *chl* (chromosome loss) (Kouprina et al. 1988, 1993), *mcm* (minichromosome maintenance defective) (Maine et al. 1984), *ctf* (chromosome transmission fidelity) (Hyland et al. 1999; Spencer et al. 1990), *ndc* (nondisjunction) (Goh and Kilmartin 1993), and *cin* (chromosome instability) (Hoyt et al. 1990) mutants in *Saccharomyces cerevisiae* (for review see Lechner and Ortiz 1996), and *dis* (defective in sister chromatid disjoining) (Ohkura et al. 1988) and *mis* (minichromosome instability) (Takahashi et al. 1994) mutants in *Schizosaccharomyces pombe* were identified. To isolate the kinetochore proteins from the mutants found, chromatin immunoprecipitation and localization studies were performed. These mutants provided the initial frame work for future work in more complex systems, such as human cell culture. Technological advances in cellular biology, microscopy, and proteomics, as well as the sequencing of genomes, have greatly improved our ability to study kinetochore proteins.

The budding yeast kinetochore is the smallest, and its assembly is the best characterized. It is composed of more than 65 constitutive proteins found in distinct subcomplexes. Based on interaction and dependency studies, the budding yeast protein complexes are classified as inner, central, or outer (for review, see Cheeseman et al. 2002). At the innermost layer of the kinetochore are proteins that directly bind the centromeric chromatin. There are 2 protein complexes that bind with sequence specifity, CBF1 at CDEI, and CBF3 at CDEIII. scCse4p, the *Saccharomyces cerevisiae* CENP-A homolog, requires the centromere binding factor 3 (CBF3) protein complex for centromere localization. The CBF3 complex consists of 4 proteins: Ndc10, Cep3, Skp1, and Ctf13. The CBF3 complex is not evolutionarily conserved and the only component that has been found to have

a homolog in higher eukaryotes is Skp1 (Connelly and Hieter 1996). There are at least 5 central and outer protein complexes: the COMA complex (De Wulf et al. 2003; Ortiz et al. 1999), the Ctf3p complex (Measday et al. 2002), the MIND/Mtw1 complex (De Wulf et al. 2003; Euskirchen 2002; Westermann et al. 2003), the Ndc80 complex (Janke et al. 2001; Wigge and Kilmartin 2001), and the Dam1 complex (Cheeseman et al. 2001; Janke et al. 2002; Li et al. 2002). Among the budding yeast kinetochore protein complexes, the MIND/Mtw1 and Ndc80 complexes are the only ones that have been found to be conserved, but additional individual proteins, such as scMif2, the CENP-C homolog, are also conserved (Kitagawa and Hieter 2001). It has been proposed that the yeast kinetochore architecture is conserved as the basic repeated subunit in higher eukaryotes (Zinkowski et al. 1991). However, there are several differences, including the fact that proteins of the yeast kinetochore are constitutive and not cell cycle regulated, as they are in higher eukaryotes.

The localization of CENP-C at the centromere is dependent on CENP-A in all organisms examined (Goshima et al. 2003; Moore and Roth 2001; Oegema et al. 2001; Van Hooser et al. 2001; Westermann et al. 2003), and also on CENP-H and CENP-I in chicken DT40 cells (Nishihashi et al. 2002; Trazzi et al. 2002). The centromere localization of CENP-H and CENP-I is dependent upon each other, CENP-A, and Mis12 (Nishihashi et al. 2002). The localization of CENP-U(50) is dependent upon CENP-H and CENP-I, and the 3 proteins coimmunoprecipate together (Minoshima et al. 2005) (see Fig. 1*a*). The complex interactions and dependencies have made determining the vertebrate centromere and kinetochore protein architecture challenging. This process is further complicated by the continuing discovery of additional proteins (Foltz et al. 2006; Okada et al. 2006).

There are 2 centromere assembly pathways, 1 through Mis12, and the other through CENP-A. In many organisms, Mis12 does not require CENP-A for localization to the centromere, and its depletion doesn't affect the localization of CENP-A (Goshima et al. 2003; Takahashi et al. 2000). In cases where the recruitment of these 2 proteins are independent of each other, neither protein alone is sufficient for assembly of a functional kinetochore (Goshima et al. 2003; Takahashi et al. 2000). These 2 pathways are not simple and linear, but branched and interconnected.

In human cells, Mis12 interacts with HP1α and HP1γ, as well as with the kinetochore proteins Zwint-1, KNL-1, and the Ndc80 and PMF1 kinetochore complexes (Cheeseman et al. 2004; Obuse et al. 2004) (see Table 1). HP1α and HP1γ are essential for recruitment of Mis12 to the centromere, and in turn, Mis12 is required for localization of Zwint-1, KNL-1, the Ndc80, and PMF1 complexes to the kinetochore (Obuse et al. 2004). This finding explains why heterochromatin is required for the assembly of a functional kinetochore, and the interaction with Mis12 may be the stabilizer of HP1 at the pericentromere boundary at mitosis. CENP-A may also be involved in the recruitment of Zwint-1, because when CENP-A is overexpressed, Zwint-1 is found at the ectopic sites with CENP-A and CENP-C (Van Hooser et al. 2001).

Not all organisms have 2 centromere assembly pathways, because the role of Mis12 is not conserved. Although in *Schizosaccharomyces pombe* and humans, Mis12 localizes to the centromere independent of CENP-A (Goshima et al. 2003; Takahashi et al. 2000), in *C. elegans,* Mis12 localization is dependent on CENP-A (Cheeseman et al. 2004). This difference may be due to structural centromeric differences; because *C. elegans* are holocentric; they do not have a single site of kinetochore assembly, but rather, numerous sites along the chromosome arms (Maddox et al. 2004). It is not known whether *C. elegans* have HP1 containing pericentromeric heterochromatin required for kinetochore formation, but if they do not, this may explain the difference.

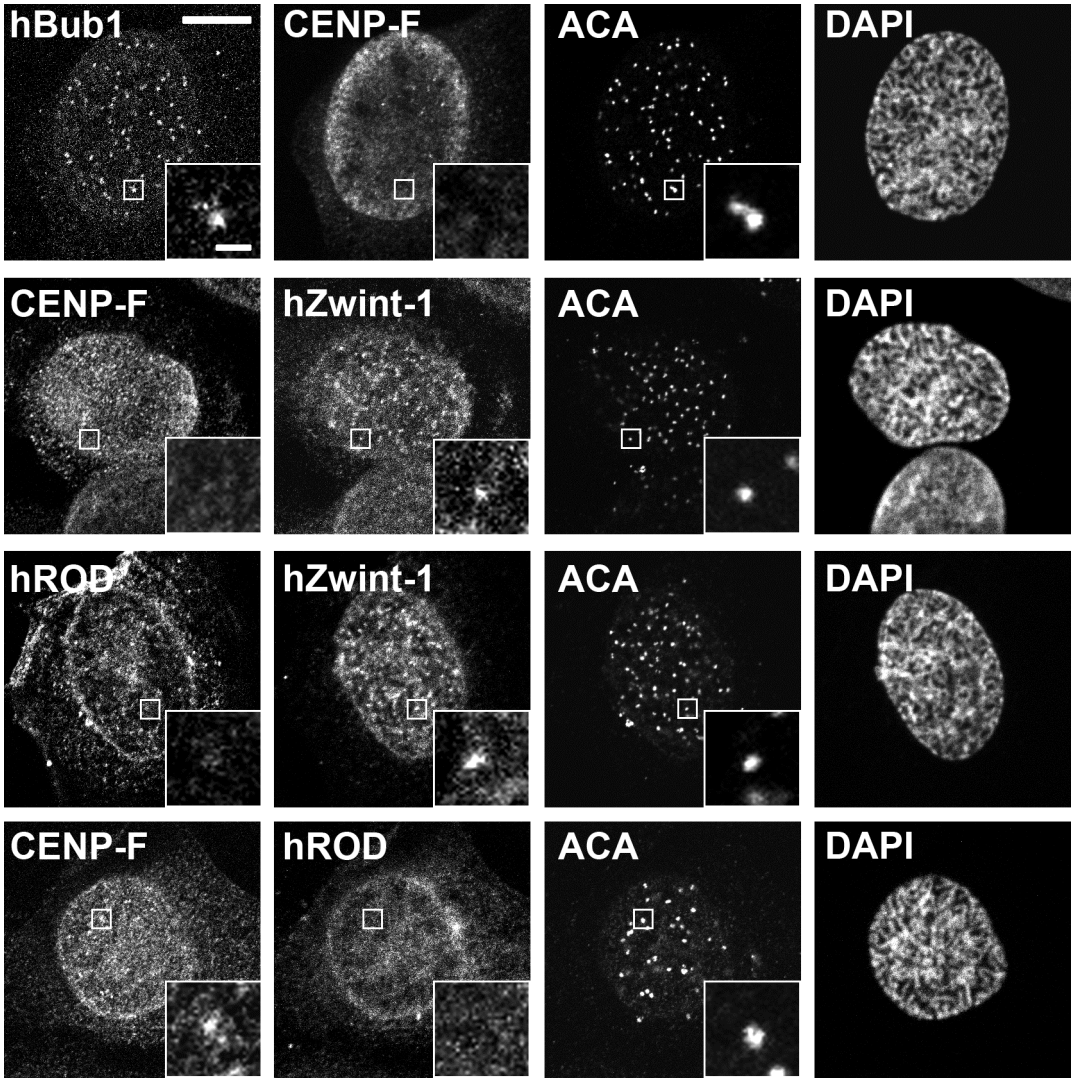
The assembly of the kinetochore upon the centromere is cell cycle dependent, and the order of protein assembly and localization dependencies have been studied for many inner and outer kinetochore proteins. Some proteins appear at the kinetochore in late G2 and early prophase, while others are not assembled until after nuclear envelope breakdown. The proteins that localize to the kinetochore early in the pathway provide the foundation for those that follow, but this is not an absolute truth because there are 2 different pathways that are not linear, and although they share some components, they also have proteins specific to that pathway. Bub1, a mitotic kinase, is localized to the kinetochore early in the assembly pathway (late G2/early prophase) prior to CENP-F, BubR1 and CENP-E (see Table 1), which arrive in this order (Chan et al. 1998; Jablonski et al. 1998), and it is required for the localization of all 3 (Johnson et al. 2004). CENP-F and CENP-E, however, do not require each other (Chan et al. 2005), and whether CENP-E requires BubR1 is organism dependent (Chen 2002; Regnier et al. 2005). In addition to the 3 aforementioned proteins, we have found that Bub1 localizes to the kinetochore prior to Zwint-1 or Rod (see Fig. 3). It is not known whether Bub1 is required for the localization of these 2 proteins, but the compiled model of assembly predicts that Bub1 will not be involved in recruiting either Zwint-1 or Rod to the kinetochore (see Fig. 1*b*). The model is by no means complete, and in many cases, the physical interactions have not been analyzed. Future work will result in the identification of additional proteins, as well as the determination of how they all work together to make a functional kinetochore.

## Conclusions and future directions

Advances in fluorescence microscopy have made it possible to analyze the 3D structural features of the centromere and stretched chromatin fibers. With these methods, it has been found that CENP-A is interspersed with histone H3 in the centromeric heterochromatin and that the amino-terminal tail of histone H3 in this region contains a distinct set of epigenetic modifications. This unique epigenetic labeling pattern at the centromere may be the missing link in the explanation of why CENP-A alone at other sites is not able to form a functional kinetochore.

Another possible explanation is based on the fact that Mis12 plays a key role in the assembly of a functional centromere. If Mis12 is not present with CENP-A at ectopic sites when CENP-A is overexpressed, then the Mis12-dependent assembly pathway would not be present, and a functional kinetochore could not be formed. It is not known whether Mis12 colocalizes with CENP-A at the random lo-

**Fig. 3.** Sequential localization of kinetochore proteins. Kinetochore proteins hBub1, hZwint-1, hCENP-F, and hROD assemble onto the kinetochore in a temporal manner. HeLa cells were synchronized by double thymidine block and harvested at time points corresponding to late G2/early mitosis (Jablonski et al. 1998). Temporal assembly of kinetochore components is observed with immunofluorescence. hBub1 and hZwint-1 localize to the kinetochore prior to CENP-F and hROD, and CENP-F localizes to the kinetochore prior to hROD. White boxes indicate single kinetochores that are enlarged in the bottom right hand corner of each image. Large scale bar = 10 $\mu$m; small scale bar = 1 $\mu$m. hBub1 was detected with a mouse monoclonal antibody and Alexa594 conjugated anti-mouse secondary antibody. CENP-F was detected with a polyclonal rabbit antibody, and Texas Red conjugated anti-rabbit secondary antibody. hZwint-1 was detected with a polyclonal rat antibody, and Cy5 conjugated anti-rat secondary antibody. hROD was detected with a polyclonal rabbit and Texas Red conjugated anti-rabbit secondary antibody in column 1, and a polyclonal rat with Cy5 conjugated anti-rat secondary antibody in column 2. Centromeres were detected with human autoimmune anti-centromere sera (ACA), which stains CENP-A, CENP-B, and CENP-C, as well as Cy2 conjugated anti-human secondary antibodies. DNA is stained with DAPI. Images were collected using a F-Flour 40X objective (NA 1.3), with a Zeiss confocal laser scanning module (LSM 510) mounted on a Zeiss Axiovert 100 mol/L inverted microscope. DAPI was excited with a laser line of 364 nm, and the signal was collected with a band-pass filter of 385–470 nm. Cy2 and Texas Red conjugated secondary antibodies were excited with laser lines of 488 nm, and collected with band pass filters of 505–550 nm and 543 nm, and a band pass filter of 560–615 nm, respectively. Cy5 conjugated secondary antibodies were excited with a laser line of 633 nm and collected with a long pass filter of 650 nm. Images from different channels were collected sequentially to avoid cross-talking of the fluorescence signal.




cations when CENP-A is overexpressed, nor is it known whether heterochromatin forms around these sites.

The independence and interdependence seen in the protein assembly at the centromere demonstrates the complex nonlinear arrangement that continues from the centromere up through the kinetochore. It is important to determine the roles of the many centromere and kinetochore proteins and strive to find additional members so that the mechanisms of centromere determination and kinetochore assembly can be fully elucidated.

While it is known that CENP-A and pericentromeric heterochromatin are essential for functional centromere and kinetochore formation in vertebrate systems, the mechanisms that affect CENP-A loading or pericentromeric heterochro-

matin establishment are less well understood in mammalian systems than they are in yeast. With advances in molecular techniques and siRNA technology, a demonstration of the involvement of proteolysis, RNAi, and base excision repair pathways in centromere determination in mammalian systems is expected.

Another aspect of the centromere that needs to be further examined is the mechanism by which the centromere position is maintained during DNA replication. It is known that epigenetic modifications are heritable, and this seems to be the most likely scenario. There is, however, the possibility that CENP-A itself is the propagation signal for the centromere during replication or that epigenetics and CENP-A cooperate to maintain the centromere identity. Whatever the mechanism, it is crucial that the cell be able to form and maintain a functional centromere from 1 mitotic division to the next.

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