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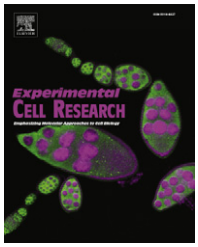

Review Article

# Replicating centromeric chromatin: Spatial and temporal control of CENP-A assembly

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ABSTRACT

The centromere is the fundamental unit for insuring chromosome inheritance. This complex region has a distinct type of chromatin in which histone H3 is replaced by a structurally different homologue identified in humans as CENP-A. In metazoans, specific DNA sequences are neither required nor sufficient for centromere identity. Rather, an epigenetic mark comprised of CENP-A containing chromatin is thought to be the major determinant of centromere identity. In this view, CENP-A deposition and chromatin assembly are fundamental processes for the maintenance of centromeric identity across mitotic and meiotic divisions. Several lines of evidence support CENP-A deposition in metazoans occurring at only one time in the cell cycle. Such cell cycle-dependent loading of CENP-A is found in divergent species from human to fission yeast, albeit with differences in the cell cycle point at which CENP-A is assembled. Cell cycle dependent CENP-A deposition requires multiple assembly factors for its deposition and maintenance. This review discusses the regulation of new CENP-A deposition and its relevance to centromere identity and inheritance.



## Contents



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## Introduction

The genetic material in eukaryotic cells is organized in a packed nucleoprotein complex, chromatin. Chromatin is comprised of nucleosomes with ~147 bp of DNA wrapped around a histone octamer that contains two molecules of H3, H4, H2A and H2B. This nucleosome organization is thought to be present along the entire chromosome, with the most conspicuous exception at the centromere, a specialized chromosomal domain necessary for the correct segregation of eukaryotic chromosomes prior to cell division [1]. This region is a complex DNA domain that in humans contains extensive tandemly repeated arrays of a 171 bp DNA sequence element called α-satellite [2]. Remarkably, although centromeric DNA is the element that insures chromosome inheritance, it has no sequence conservation species to species. A common feature of centromeres, despite size and sequence divergence across species, is that in centromeric chromatin from yeast to man the canonical nucleosome histone H3 is replaced by a centromere specific variant initially identified in humans and named CENP-A [3,4]. Homologues have now been identified in many other species (e.g., CID in *Drosophila melanogaster*, Cnp1 in *Schizosaccharomyces pombe*, and Cse4 in *Saccharomyces cerevisiae*).

Not surprisingly, CENP-A incorporation at centromeric nucleosomes is tightly controlled. Artificially targeting of CENP-$A^{CID}$ to a new location in *Drosophila* cells correlates with ectopic centromere deposition and kinetochore assembly, including microtubule-binding proteins and spindle assembly checkpoint proteins (Ndc80 and Mad2); this leads to formation of multicentric chromosomes that are consequently mis-segregated at high frequency [5,6]. Together with the discovery in humans of neocentromeres (the stable acquisition of a new centromere at a new chromosomal site without any DNA sequence change) [7,8] stably bound by CENP-A [9], the current evidence supports a model in which CENP-A chromatin rather than DNA sequence is the major determinant of mammalian centromere identity.

The question of how CENP-A can epigenetically define centromeres remains unsolved. One initial proposal is that it can induce a conformationally more constricted nucleosome structure that serves to template its own replication and nucleate recruitment of the ~50 other centromeric components. Evidence for this initially came from deuterium exchange/mass spectrometric analysis, in which exchange of the amide proton on each peptide bond with protons in solution provides a direct measure of accessibility to solvent. Buried residues that are inaccessible to solvent – and which remain inaccessible despite transient flexing driven by thermal energy – are easily detected by absence of (or very slow) proton exchange with tritium in solution. This approach revealed that CENP-A nucleosomes are more rigid than their counterpart histone H3-containing nucleosomes — with many positions exchanging amide protons > 10 fold more slowly.

**Table 1 – Timing of CENP-A loading across species.**

| Organism | CENP-A cell cycle-dependent deposition | References |
|---|---|---|
| Human cells | Late telophase-early G1 [a] | [21] |
| *Drosophila melanogaster* embryos/S2 cells | Anaphase/metaphase [b] | [26,27] |
| *Xenopus laevis* | Mitotic exit-early interphase [c] | [24,25] |
| DT40 chicken cells | Mitotic exit | [23] |
| *S. pombe* | S-phase (and G2 phase) [d] | [30] |
| *S. cerevisiae* | S-phase [e] | [29] |
| *Arabidopsis thaliana* | G2 phase | [28] |

[a] HJURP localizes to centromere in telophase just prior CENP-A assembly, hMIS18α in anaphase [35,36,43,45].
[b] CAL1 is recruited to centromere during prophase [27].
[c] xHJURP localizes at centromere in mitosis, M18BP1 during metaphase [24,25].
[d] Scm3 and Mis18 complex localize to centromere at all cell cycle phases except from metaphase to mid-anaphase [40,42,46].
[e] Scm3 is present at centromere at all cell cycle phases [47].

This rigidity/conformational inflexibility initiates within the CENP-A/histone H4 pre-nucleosomal heterotetramer [10–12] and can be transferred to histone H3 by a 22 amino acid exchange of the loop 1 and α2 helix of CENP-A into the corresponding domain of histone H3 [11,12]. This short domain has been called the *CENP-A targeting domain* (CATD) because when substituted into histone H3 [11] it is sufficient to convert H3 into a centromere targeting histone where it can maintain centromere function when CENP-A levels are lowered [13].

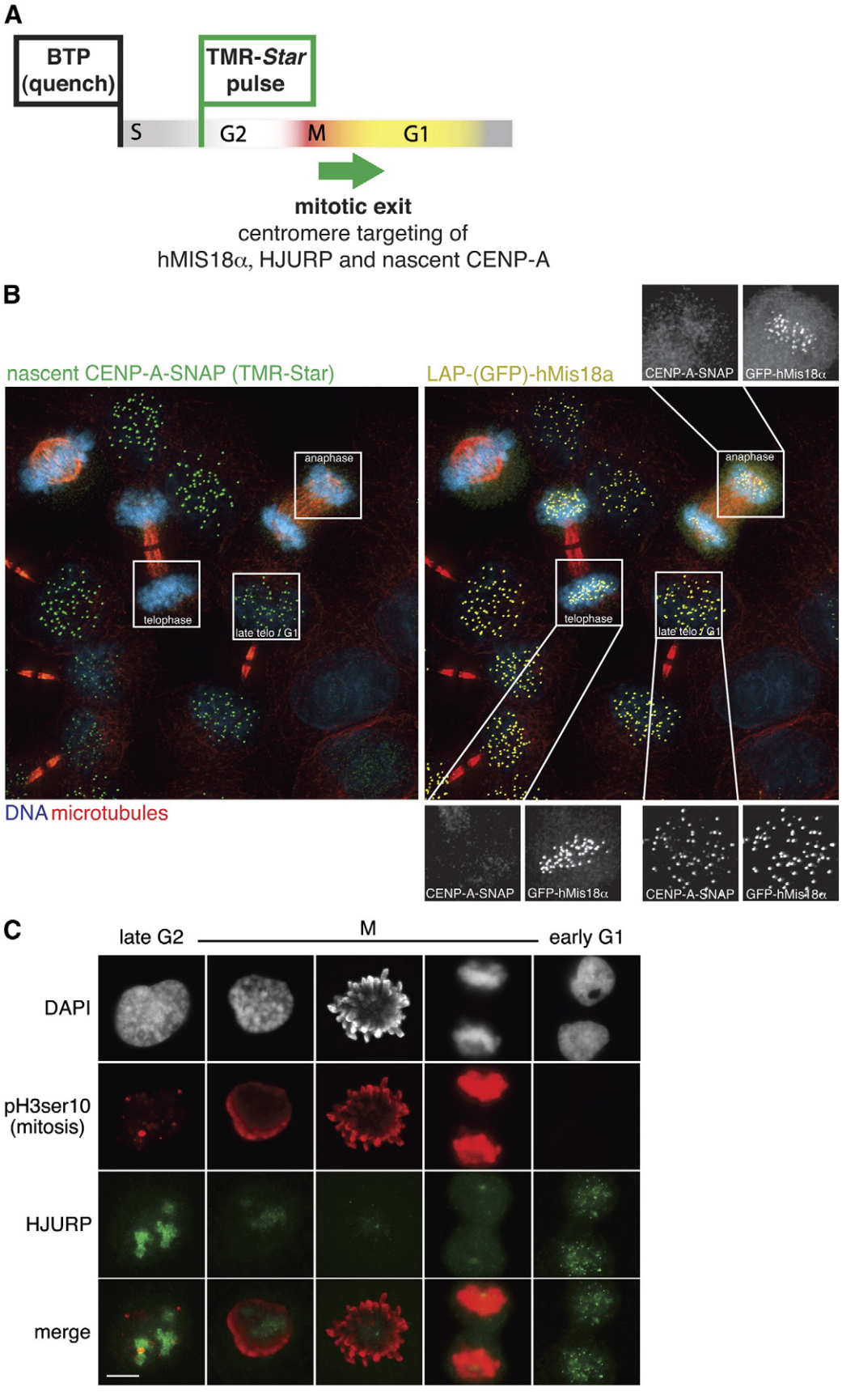
A crystal structure of the CENP-A/histone H4 heterotetramer confirmed its structural compactness [10], with a 9–14° skewing of the CENP-A dimer (relative to the corresponding domains of histone H3 in the H3/H4 heterotetramer). Nevertheless, a subsequent crystal structure of the full CENP-A nucleosome containing a histone octamer did not reveal the same compactness of the CENP-A nucleosome relatively to the canonical H3 nucleosome [14]. The crystal structure is, of course, a static view that does not assay conformational dynamics. Indeed, despite the similarity in final structure, it should be emphasized that in solution the CENP-A-containing nucleosomes are substantially more conformationally constrained — especially in the α-helix 1 and 2, as revealed by deuterium exchange/mass spectrometry [12]. Lastly, an insight from the crystal structure is that Loop1 in the CATD domain protrudes from the CENP-A nucleosome (and heterotetramer), positioned appropriately for it to be able attract additional centromeric components [10,14].

Beyond a structurally altered nucleosome – with the exposed Loop 1 – an alternative, but not mutually exclusive, possibility for CENP-A chromatin is that CENP-A induces changes in the higher-

**Fig. 1 – hMis18α, HJURP and CENP-A assemble at centromeres in a cell cycle-dependent manner. (A) Outline of the SNAP tagging experiments in (B) to establish temporal order of Mis18 recruitment to centromeres at mitotic exit and CENP-A loading. (B) Mis18 recruitment to centromeres precedes new CENP-A assembly at mitotic exit, as demonstrated with synchronized HeLa cells stably expressing CENP-A-SNAP. LAP-hMis18α is targeted to centromere in anaphase, prior to the late telophase/G1-CENP-A-SNAP assembly [adapted from [45]]. (C) HJURP localization at centromere occurs after mitotic exit, as determined with indirect immunofluorescence for phospho-histone H3 and HJURP [adapted from [35]].**

order chromatin structure as it modifies the entry/exit of the DNA from the nucleosome [15], potentially producing a more condensed chromatin state [14,16].

CENP-A chromatin may also be marked by a structure that differs from the classical canonical octameric H3-nucleosomes. Recent studies offer conflicting evidence for the structure of CENP-A-



containing chromatin [17]. The different proposals include, among others, a classical octameric CENP-A nucleosome with two copies of each histone, a tetrasome lacking H2A:H2B dimers, a hemisome with one copy of each histone, and involvement of the budding yeast Scm3 in the final structure either as a hexasome or as a trisome of CENP-A$^{Cse4}$, H4, and Scm3. These different structures of CENP-A chromatin may represent a process of cell-cycle-dependent maturation of centromeric chromatin that is a result of the separation between centromeric DNA replication and CENP-A deposition (discussed below). Whether this is indeed the case is yet to be established. However, such a model of chromatin maturation could provide a basis for the stable inheritance of centromere identity.

## CENP-A deposition: only once at the right time

A very surprising feature of centromeric chromatin is that its replication in metazoans is uncoupled from centromeric DNA replication. Indeed, once per cell cycle assembly of CENP-A at active centromeres is a highly conserved feature of centromeres in species spanning from fission yeast to man, albeit the cell cycle position during which centromeric chromatin is replicated is different in different species (Table 1). In human cells, centromeric DNA is replicated in mid-to-late S-phase [18], while synthesis of CENP-A increases in G2 [19,20]. Pulse-chase fluorescent labeling based on SNAP-tagging has demonstrated that new CENP-A loading occurs only once per cell cycle in telophase/early G1 and requires passage through mitosis (Figs. 1A, B) [21], a result confirmed by photobleaching experiments [22]. Similar to the human findings, in chicken DT40 cells new CENP-A loading is prevented by blocking advance past anaphase with microtubule drugs [23] (Table 1). So, too, for frogs, as has been shown with *Xenopus* egg extracts in which CENP-A assembly occurs in a short time window following mitotic exit (Table 1)[24,25].

Quantitative fluorescence measurements in *Drosophila* syncytial embryos revealed that GFP-CID is rapidly assembled at centromeres during or after anaphase [26]. More recent, higher resolution quench-chase-pulse experiments using SNAP-tagging in a pair of *Drosophila* cell lines (S2 and Kc167 cells) have revealed that new CENP-A$^{CID}$ incorporation at centromeres initiates during metaphase (Table 1) [27]. Loading may require degradation of cyclin A, since blocking the proteasome by MG132 treatment or using a non-degradable form of cyclin A reduced CENP-A$^{CID}$ loading prior to anaphase onset [27].

Overall, while the precise cell cycle position of loading differs between flies and the other animal species studied, the common feature for CENP-A loading across different species of higher eukaryotes is that it occurs independent of DNA replication. Moreover, in these species, cells undergo mitosis with only half the maximal CENP-A content loaded at centromeres. All these findings suggest that passage through mitosis is a prerequisite for new CENP-A loading and assembly at the centromere.

Contrary to the animal species described above, CENP-A$^{CENH3}$ loading in *Arabidopsis thaliana* has been reported to occur in late G2 (Table 1) [28], as demonstrated by measuring the relative proportions of CENP-A$^{CENH3}$ immunostaining in different phases of the cell cycle. In single cell eukaryotes, the timing of assembly of the CENP-A homolog apparently correlates with the timing of DNA replication as it does for the canonical histones. Photobleaching experiments in budding yeast are consistent with Cse4-GFP assembly occurring mainly early during DNA replication [29]. A similar result was observed in fission yeast where incorporation of CENP-A$^{Cnp1}$ occurs predominantly during S-phase (although 25% of new CENP-A$^{Cnp1}$ loading occurs during late G2) and the timing of this replication-dependent deposition requires the cell cycle regulated GATA-type transcription factor Ams2 [30]. Indeed, in *Δams2* deficient cells the cell cycle-dependent loading of CENP-A$^{Cnp1}$ shifts almost completely to G2 [30] (summarized in Table 1). This difference between lower and higher eukaryotes in timing of CENP-A incorporation correlates with a shift in centromere DNA replication timing in S-phase, since yeast centromeres are replicated very early during S-phase [31,32] while human and *Drosophila* centromeres are replicated at mid-late S-phase [18,33]. Indeed, in the yeast *Candida albicans* stable and heritable neocentromeres are associated with a shift in replication timing (from late to early in S-phase) [34].

## CENP-A assembly factors

Similar to the canonical histone H3 that is deposited on chromatin by a series of chaperones, CENP-A loading at centromeres is mediated by factors that chaperone the loading process. Two independent affinity purification studies in humans identified a pre-nucleosomal (non-chromatin associated) CENP-A complex that consists of CENP-A, HJURP, histone H4 and nucleophosmin 1 (NPM1) [35,36]. In one of those efforts [35], an additional component was also present — RbAp48, a previously known component of both the CAF-1 complex responsible for loading the replication dependent histone H3.1 and the HIRA complex responsible for facilitating the loading of histone H3.3. The role for NPM1 in CENP-A loading is not established and unlikely to be unique for CENP-A, as it binds ATP and has been implicated as an ATP-dependent chromatin remodeler/chaperone for both H3:H4 and H2A:H2B and multiple other cellular roles [37,38].

HJURP (Holliday junction recognizing protein) was demonstrated to act as the CENP-A chaperone protein that binds directly to soluble CENP-A and is required for its chromatin assembly [35,36]. *Xenopus* HJURP was also shown to have characteristics of a CENP-A loader, as adding exogenous xHJURP to egg extracts results in substantial assembly of new CENP-A into centromeric chromatin [24]. HJURP contains a region of homology to the yeast Scm3 [39] and both appear to function similarly; depletion of HJURP from human cells [35,36] or from *Xenopus* egg extracts [25] causes defects in CENP-A assembly similar to the defects resulting from Scm3 mutation in yeast [25,35,36,40]. Furthermore, tethering HJURP to an ectopic non-centromeric locus is sufficient to induce incorporation of CENP-A into the chromatin at this site and results in creating a functional de novo kinetochore [41].

The targeting of CENP-A and HJURP to centromeres is dependent on another group of proteins, the Mis18 complex, initially identified in a study of chromosome mis-segregation mutants in fission yeast and which when mutated lead to the loss of CENP-A$^{Cnp1}$ from centromeres [42]. The human homologue for the fission yeast mis18 is a complex comprised of three components, Mis18-α, Mis18-β and M18BP1. Depletion of any of the three subunits by RNAi rapidly abolishes the centromere recruitment of newly synthesized CENP-A, followed by defects such as misaligned chromosomes, anaphase mis-segregation and

interphase micronuclei [43]. Depletion of *Caenorhabditis elegans* M18BP1$^{KNL-2}$ from *C. elegans* embryos [44] or xM18BP1 from *Xenopus* egg extracts [24] also prevents assembly of CENP-A at centromeres. Furthermore, in cells depleted of the Mis18 complex, HJURP fails to localize to centromeres [41].

Since both the Mis18 complex and HJURP are necessary to achieve spatial and temporal control of CENP-A nucleosome assembly, a key goal ahead is to decipher the regulatory signals that control their centromeric localization. In human cells, HJURP is localized to centromeres during late anaphase/telophase (Fig. 1C) and remains associated with centromeres during G1, the time window during which new CENP-A assembly occurs [35,36]. The human M18BP1 complex localizes to centromeres at anaphase, just before HJURP, and remains associated until mid G1 (Figs. 1A, B) [43,45]. Similar to human cells, in *Xenopus laevis* xHJURP localizes at centromeres in mitosis [25] and M18BP1 during metaphase just prior to CENP-A loading [24,25]. In *Drosophila*, CAL1 has been hypothesized to have similar function to HJURP and M18BP1, despite the lack of sequence homology. Accordingly, similar to HJURP/M18BP1 cell cycle-dependent loading, CAL1 associates with centromeres in prophase just before CENP-A$^{CID}$ loading [27].

These results led to the proposal that the Mis18 complex "primes" the centromere and prepares it for the loading of new CENP-A [41], perhaps through histone H4 acetylation [41,43]. Surprisingly, in yeast, Scm3 and the Mis18 complex seem to remain associated during all the stages of the cell cycle, excluding a short time from metaphase to mid-anaphase in fission yeast [40,42,46,47]. Despite their suggestive localization pattern and clear role in CENP-A assembly, none of the yeast or human Mis18 proteins appears to bind CENP-A directly [42,43] and the process by which the Mis18 complex is recruited to centromeres during mitosis is unknown.

## Regulation, stabilization and maintenance of newly incorporated CENP-A

Cell cycle-dependent loading of CENP-A at centromeres is now recognized to require the constitutive centromere protein CENP-C. Straight and colleagues have recently shown that CENP-C directly interacts with M18BP1 in both *Xenopus* and humans [24]. Further, since CENP-C binds CENP-A nucleosomes directly [48] and is required for new CENP-A assembly [48,49], the interaction between CENP-C and M18BP1 creates a molecular bridge between CENP-A and M18BP1. Furthermore, depletion of CENP-C resulted in disruption of centromere targeting of both M18BP1 and HJURP during late mitosis, leading to inhibition of new CENP-A assembly in G1 [24]. Thus, a key role of CENP-C is the recruitment of M18BP1 to centromeres as a second means to promote CENP-A assembly.

It should be emphasized that the mitotic trigger that initiates centromere propagation remains unresolved. Nevertheless, work from the Jansen group has established that the CENP-A assembly process is inhibited by Cdk activity [23]. Treatment of G2-synchronized HeLa cells with the pan-Cdk inhibitors roscovitine or purvalanol resulted in premature centromeric CENP-A assembly in 50% of the G2 cell population accompanied by a rapid recruitment of Mis18-α and M18BP1 to centromeres prior to the assembly of CENP-A. Similarly, DT40 chicken cell lines lacking active Cdk1 and Cdk2 induce CENP-A assembly during G2, indicating that both Cdk1 and Cdk2 function to inhibit premature CENP-A loading prior to mitotic exit. Moreover, the machinery responsible for CENP-A assembly is present already in S-phase but is normally inhibited primarily by Cdk2 activity.

Lastly, premature centromere recruitment of M18BP1 is prevented by its phosphorylation (by a kinase yet to be determined), which also occurs in a cell cycle dependent manner — peaking at mitosis. Upon mitotic exit M18BP1 is dephosphorylated and only the unphosphorylated form of M18BP1 can then be rapidly targeted to centromeres to initiate the process of CENP-A replenishment.

## Stabilizing newly replicated centromeric chromatin in G1

What happens after nascent CENP-A is deposited at mitotic exit and early G1? Recent studies have pointed to several factors that may have important roles in centromeric chromatin maturation in G1. Rsf-1 and SNF2h are two subunits of the ATP-dependent chromatin remodeling and spacing factor RSF. These two proteins were found along the components in CENP-A affinity precipitates from interphase extracts [50,51]. Native chromatin immunoprecipitation (nChIP) and immunoprecipitation experiments by Yoda and colleagues showed that unlike the Mis18 proteins, Rsf-1 and SNF2h associate with CENP-A chromatin at the mononucleosome level [52]. Rsf-1 is localized at centromeres in mid-G1 and is retained at centromeres for a period of 4 h. Thus, Rsf-1 centromere localization occurs several hours after the recruitment of the Mis18 complex and the deposition of new CENP-A.

Interestingly, depletion of Rsf-1 using siRNA resulted in normal CENP-A levels at centromeres, but this centromeric CENP-A became sensitive to high salt extraction and was removed easily from the chromatin core following a 0.6 M NaCl salt wash. These results suggest a model in which CENP-A is initially targeted to centromeres in late M/early G1 with relatively weak association to the chromatin, after which it is incorporated and assembled into stable core chromatin through the RSF remodeling and spacing function. Future experiments are still needed to further determine whether the salt sensitive pool observed indeed represents newly targeted CENP-A.

Following incorporation into core chromatin, newly assembled CENP-A molecules are apparently further stabilized by the action of MgcRacGAP [53]. MgcRacGAP was previously known to exhibit GAP activity for three Rho family small GTPases (RhoA, Rac1 and Cdc42) [54] and to be a part of the centralspindlin complex that functions during cytokinesis and localizes to the spindle midzone following chromosome segregation [54,55]. Added to this, MgcRacGAP has been previously shown to co-purify with CENP-A chromatin [51,52].

A role for MgcRacGAP in regulation of centromere chromatin has recently emerged. Using immunoprecipitation experiments, Maddox and colleagues have shown that MgcRacGAP co-purifies with M18BP1 [53]. Depletion of MgcRacGAP using shRNA resulted in the loss of only the newly incorporated-CENP-A at centromeres, without any effect on M18BP1. Quantitative live cell measurements revealed that MgcRacGAP localizes to centromeres only at late G1 (~10 h after anaphase) after CENP-A levels at centromeres had doubled. Furthermore, the authors suggested that MgcRacGAP functions together with its GEF partner, ECT2, to target the small

GTPase Cdc42, since depletion of either one resulted in loss of centromeric CENP-A. The downstream targets of Cdc42 that regulate centromeric chromatin are still to be identified. Nevertheless, these localization patterns suggest that MgcRacGAP transiently activates a Cdc42-mediated switch at centromeres to maintain newly incorporated CENP-A.

## A multi-step model for centromeric chromatin replication

Taken all together, the flurry of recent findings has led to a model in which a multi-step pathway controls the location and timing of human CENP-A nucleosome assembly (Fig. 2). During S-phase and G2, Cdk1 and Cdk2 activities are up-regulated and are essential for the initiation of DNA replication and entry into mitosis [56]. Cdk1/2 activities at these phases lead to phosphorylation of M18BP1 (and likely other proteins as well, yet to be identified) and prevent targeting of the Mis18 complex to centromeres. Mitotic exit is triggered by degradation of cyclin B, the Cdk1 activator, leading to loss of Cdk1 activity, thereby permitting association of unphosphorylated M18BP1 to centromeres as an initiating step in the process of CENP-A replenishment. Recruitment of the Mis18 complex to the centromere requires binding of M18BP1 to CENP-C and provides the licensing step that primes each centromere for CENP-A loading. Following priming and licensing, HJURP is recruited to the centromere while providing chaperone function for soluble CENP-A subunits.

Loading of new CENP-A subunits by HJURP takes place from late telophase to early-mid G1. Further maturation of this newly assembled centromeric chromatin occurs slowly through the transient recruitment of the chromatin remodeling and spacing factor complex RSF at mid G1, contemporaneous with removal of HJURP and the Mis18 complex. Further on at late G1, a GTPase switch at centromeres by MgcRacGAP-ECT2 is used to further stabilize newly assembled CENP-A-containing chromatin.

Several possibilities may explain the complexity of the CENP-A assembly process. First, following mitotic exit there is a need to epigenetically propagate the centromere before the next round of DNA replication. This process may take time since it involves a large number of proteins and multiple assembly and disassembly events in order to assemble and stabilize CENP-A nucleosomes in a precise manner [57]. Second, the multi-step pathway may present opportunities for quality control, especially in the stabilization phase by centromeric-localized MgcRacGAP, with the extended time and contributions from multiple components allowing promiscuous CENP-A misincorporated at non-centromeric loci to be

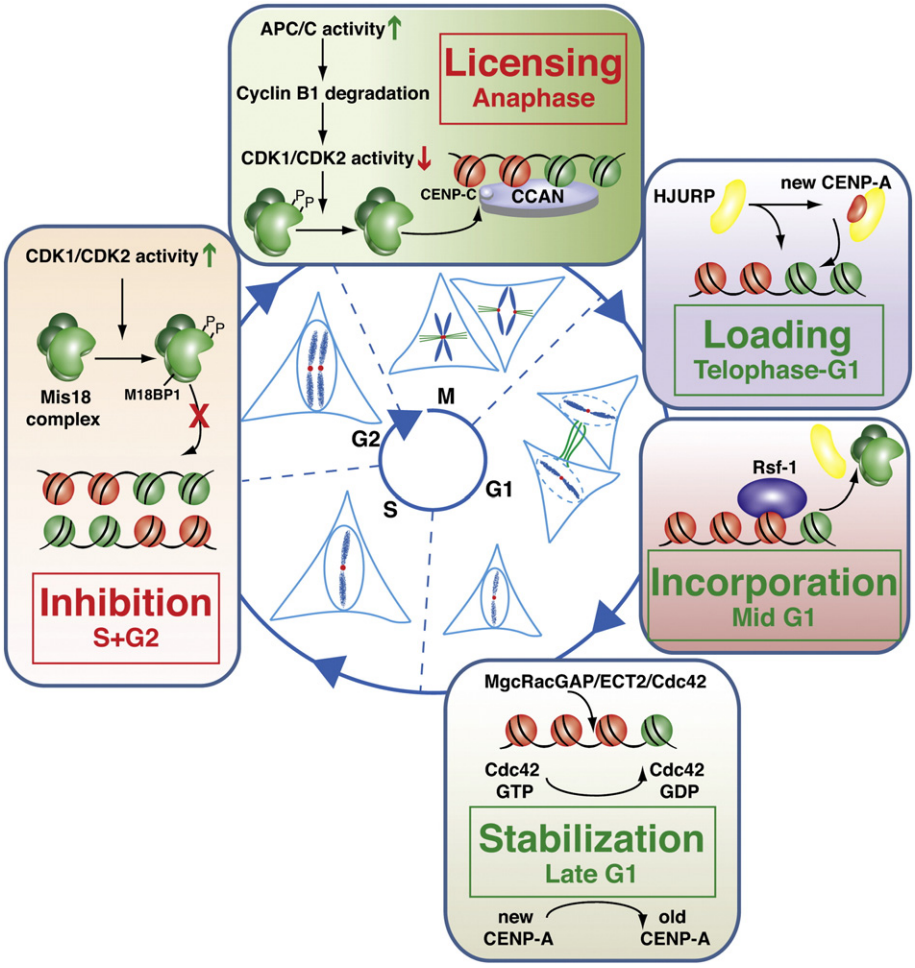



**Fig. 2 – Model of the multi-step pathway of CENP-A-containing centromeric chromatin replication utilizing sequential, temporally separated steps across the mammalian cell cycle. See full description in the main text.**

removed by mechanisms that perhaps recognize weakly associated "new" CENP-A which has not been stabilized by MgcRacGAP [53].

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