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Posttranslational mechanisms controlling centromere function and assembly

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Abstract

Accurate chromosome segregation is critical to ensure the faithful inheritance of the genome during cell division. Human chromosomes distinguish the location of the centromere from general chromatin by the selective assembly of CENP-A containing nucleosomes at the active centromere. The location of centromeres in most higher eukaryotes is determined epigenetically, independent of DNA sequence. CENP-A containing centromeric chromatin provides the foundation for assembly of the kinetochore that mediates chromosome attachment to the microtubule spindle and controls cell cycle progression in mitosis. Here we review recent work demonstrating the role of posttranslational modifications on centromere function and CENP-A inheritance via the direct modification of the CENP-A nucleosome and pre-nucleosomal complexes, the modification of the CENP-A deposition machinery and the modification of histones within existing centromeres.

Epigenetic specification of centromeres

Chromosome segregation during mitosis and meiosis is orchestrated by the centromere. Centromeres are unique chromosome domains that direct the assembly of the kinetochore during mitosis, which mediates microtubule attachment, checkpoint signaling and chromosome movement [1]. Centromeres are defined by the presence of the histone H3 variant Centromere Protein A (CENP-A).

DNA sequence is sufficient to determine centromere location in budding yeast; however, centromere identity in most higher eukaryotes is not dependent on DNA sequence. In most higher eukaryotes, centromere specification relies on the incorporation of CENP-A nucleosomes, and the location of CENP-A nucleosomes is sufficient to specify the site of centromere formation [2]. Many neocentromeres have been identified, where centromere proteins and function have been relocalized to a new site within the chromosome devoid of alpha-satellite sequence [3]. Centromeres are therefore defined by the epigenetic

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mechanisms that facilitate CENP-A deposition and stable inheritance across the cell cycle, and across multiple generations, all of which are critical for maintaining centromere identity.

Covalent posttranslational modification (PTM) of histones is a major mechanism to regulate chromatin function. Here we review recent advances in our understanding of how PTMs of CENP-A, canonical histones within the centromere, and proteins involved in CENP-A deposition regulate centromere specification. We have focused our review on the human centromere; however, several examples of PTMs in important model systems are also included.

Intrinsic features of CENP-A modifications and CCAN recruitment

Posttranslational modifications of histones control chromatin function through intrinsic effects on chromatin structure, as well as the selective recruitment of proteins. CENP-A undergoes different types of PTMs in the amino terminal tail and within the histone fold domain (Figure 1a). Several modifications of CENP-A contribute to the recruitment of the CCAN (constitutive centromere associated network) (Figure 1a,b). The first PTM of human CENP-A identified was phosphorylation of Ser7, based on its similar location with H3 amino terminal tail phosphorylation at Ser10 [4]. Mutation of this site leads to errors in chromosome segregation and cytokinesis [5,6]. Ser7 phosphorylation is mediated by the Aurora kinases, and has been shown to indirectly recruit CENP-C through phospho-binding protein 14-3-3 [4–7]; although, the primary direct binding site for CENP-C is the carboxy terminus of CENP-A [8,9].

More recently, CENP-A was shown to be phosphorylated at two highly conserved residues, Ser16 and Ser18, both prior to its deposition and in the CENP-A nucleosome [10] (Figure 1a). CENP-A Ser18 is a substrate for cyclinE1/CDK2 phosphorylation, and is cell cycle regulated [11]. Loss of phosphorylation or hyper-phosphorylation at these sites, lead to chromosome missegregation [10,11]. *In vitro* studies suggest that CENP-A has the ability to form a salt-bridged secondary structure through intramolecular association, which is dependent on phosphorylation of Ser16/18, and therefore this dual phosphorylation may impact the higher order chromatin organization of the centromere [10].

The initiating methionine of CENP-A is removed and the exposed glycine (Gly1) residue is trimethylated on the alpha-amino group by the enzyme NRMT1 [10,12••] (Figure 1a). Amino-terminal trimethylation of CENP-A is required for full recruitment of the CENP-T and CENP-I proteins of the CCAN, but does not affect CENP-C binding [12••] (Figure 1b). *S. pombe* centromeres show a similar specific requirement for the SpCENP-A amino terminus in CENP-I and CENP-T recruitment [13]. Loss of CENP-A alpha-amino-terminal methylation leads to chromosome missegregation and abnormal microtubule spindles.

Modification of CENP-A also occurs within the histone fold domain. Lys124 in the α3 helix of CENP-A has been shown to undergo methylation and acetylation, as well as ubiquitylation (discussed below) [14–16] (Figure 1a,c). Molecular dynamics simulations suggest that acetylation of the Lys124 may induce conformation changes in the CENP-A

nucleosomes resulting in limited access of the C-terminal tail and thus inhibition of CENP-C binding.

Proteolytic degradation of non-centromeric CENP-A is a potential mechanism to restrict CENP-A to centromeres and eliminate incorrectly or ectopically deposited CENP-A nucleosomes. The ubiquitylation-dependent proteolytic degradation of CENP-A$^{Cse4/CID}$ is clearly established in yeast and *Drosophila*; although the pathways in human cells are still unknown. So far, four E3 ubiquitin ligases have been reported to mediate CENP-A^{Cse4} ubiquitylation. These include Psh1, Rcy1, Slx5 and Ubr1 [17–19,20•,21•,22,23]. CENP-A^{Cse4} is protected from degradation at the centromere, while CENP-A^{Cse4} in chromosome arms is degraded. Notably, Slx5 mediated ubiquitylation is dependent on SUMOylation of CENP-A^{Cse4} [21•], suggesting that CENP-A^{Cse4} degradation may be under complex regulatory control. *Drosophila* dCENP-A^{CID} protein levels are regulated by the F-Box Protein Partner of Paired (Ppa), a component of the SCF E3-ubiquitin ligase complex [24]. E3 ligases regulating the stability of human CENP-A have not yet been identified.

Modifications of the CENP-A nucleosome that are present within the histone H4 tail also contribute to CENP-A nucleosome function. Monomethylation of Lys20 on the histone H4 tail within the CENP-A nucleosome has been observed in chicken cells [25] (Figure 1b). The removal of this mark by targeting an H4 Lys20 demethylase to the centromere leads to errors in centromere formation and chromosome segregation.

Pre-nucleosomal posttranslational modification of centromeric histones

Histones associate with a series of chaperone proteins to facilitate their assembly into nucleosomes [26,27]. The histone H3.1 and H3.3 variants differ by only a few amino acids (96% identity), whereas CENP-A is more divergent (~50% identity). Based on these differences, the histone H3 variants interact with distinct chaperones that determine the timing and the location of their deposition in chromatin (Figure 2a). HJURP (Holliday junction recognition protein) is the CENP-A specific histone chaperone. The CENP-A binding domain of HJURP is conserved from budding yeast to humans which is essential for the deposition of new CENP-A [28–30]. *Drosophila* has evolved a non-homologous CENP-A chaperone, CAL1 [31,32], and some organisms, including *C. elegans* lack a clear HJURP or CAL1 homolog, suggesting these organisms have evolved different, but potentially related mechanisms to establish centromeric chromatin.

HJURP and the yeast homolog Scm3 interact with the heterodimer that includes a single CENP-A and histone H4 [33,34] (Figures 1c and 2b). Posttranslational modification of the histone H4 subunit is required for proper deposition of the CENP-A nucleosome. RbAp46 and RbAp48 subunits found in many chromatin assembly complexes are associated with pre-nucleosomal CENP-A/H4 and HJURP [29,35,36••] (Figure 2b). Mis16, the *S. pombe* homolog of RbAp46/48, recognizes both CENP-A and histone H4 in the heterodimer [37]. RbAp46/48 recruits HAT1 (histone acetyltransferase 1) to the hetero-dimer and acetylates Lys5 and Lys12 on histone H4. In this respect, the modification of the preassembled CENP-A/H4 dimer is similar to histone H3/H4, which is also acetylated on the same residues of histone H4 [26]. Depletion of RbAp46/48/Mis16 in several organisms, or the mutation of

histone H4 Lys5 or Lys12 residues to non-acetylated amino acids, reduces new CENP-A deposition [36••,38,39]; implying that the modification of the H4 subunit of the pre-nucleosomal heterodimer is essential for CENP-A deposition.

Posttranslational modification of CENP-A is emerging as a possible mechanism to regulate HJURP binding and thus CENP-A deposition. However, contradictory experimental evidence for the roles of CENP-A PTMs in this process means that the importance of the regulatory step remains somewhat murky. The ubiquitin ligase CUL4 in complex with RDX1-COPS8 or DDB1 has been shown to be important for CENP-A nucleosome deposition, without leading to CENP-A degradation [14,40,41]. Ubiquitylation of Lys124 of CENP-A is proposed to be required for CENP-A binding to HJURP. Biochemical evidence suggests that the addition of monoubiquitin to CENP-A Lys124 promotes HJURP binding, and Lys124 mutants fail to bind HJURP and reduce new CENP-A deposition [14,41]. While Lys124 does not lie within the CATD, which is sufficient for CENP-A deposition [42], the size of the ubiquitin moiety could bridge the distance and contribute to HJURP binding. Despite the intriguing biochemical data, gene replacement experiments show that mutant CENP-A that cannot be ubiquitylated, can completely replace the endogenous CENP-A and support cell viability [43]. Therefore, direct ubiquitylation of CENP-A by CUL4 does not play an essential role in CENP-A deposition; although, we cannot preclude that Lys124 modification may play important roles under certain cellular conditions. The *Drosophila* dCENP-A^{CID} is also subjected to ubiquitylation, which facilitates its centromeric deposition [44]. CAL1 also serves as an adaptor for CENP-A^{CID} ubiquitylation by directly associating with the ubiquitin ligase Cul3/RDX1 complex.

CENP-A undergoes several other modifications prior to deposition into chromatin. The amino terminus of pre-nucleosomal CENP-A in humans is already methylated on the alpha-amino group of Gly1 by NRMT1 and phosphorylated on Ser16/18 (Figure 2b). As discussed above, both of these modifications persist after CENP-A is deposited into chromatin [10]. CENP-A is also phos-phorylated on Ser68, within the histone fold domain in a cell cycle specific manner, and is discussed below. *Drosophila* dCENP-A^{CID} is phosphorylated at Ser75/77, a site that may be analogous to Ser16/18 in human CENP-A and was enriched in the nuclear fraction [39]. In contrast, dCENP-A^{CID} is acetylated on Lys105 selectively in the prenucleosomal complex. This suggests a potential role for differential modifications in controlling distinct steps in processing prenucleosomal CENP-A.

Cell cycle control of centromere inheritance

The location of the centromere is highly fixed on the chromosome, with new CENP-A nucleosomes assembled near the domain of existing CENP-A [45]. The selective deposition of new CENP-A at the existing centromere is achieved by coupling the proteins required for CENP-A deposition to CCAN proteins, including CENP-A itself. The Mis18 complex binds HJURP directly and is required for the recruitment of HJURP and new CENP-A to the centromere in G1-phase [38,46–50] (Figure 3a). The human Mis18 complex forms an oligomeric structure that includes Mis18α and Mis18β paralogs, and Mis18BP1 [51••,52••]. CENP-C has been shown to participate in recruiting the Mis18 complex proteins to the existing centromere; although, it is unclear whether this interaction fully accounts for Mis18

recruitment [53–55]. More recently, a direct interaction between Mis18BP1 and CENP-A has been identified in *Xenopus* and chickens [56,57]. Whether direct binding of the CENP-A nucleosome by the Mis18BP1 plays a role in human centromere assembly is not yet clear.

Deposition of new CENP-A nucleosomes in human cells is restricted to early G1, immediately following mitosis, and the restricted timing involves both negative and positive regulation of the deposition pathway [58]. CDK1/2 negatively regulates CENP-A deposition in G2 and mitosis [59] (Figure 3a). Pharmacological inhibition of CDKs or the genetic knockout of CDK1 and CDK2 leads to the mis-timed loading of CENP-A in G2 phase.

The targets of CDK1 phosphorylation include CENP-A and proteins within the deposition pathway, including HJURP and Mis18BP1. CDK1 phosphorylates CENP-A at Ser68, and the degree of Ser68 phosphorylation peaks in mitosis, coincident with maximal CDK1 activity [60]. Phosphomimetic mutants result in a reduction of new CENP-A deposition, and show a decreased association with HJURP [34,60]. These data suggest that CDK1 mediated phosphorylation of CENP-A during G2-phase and mitosis contributes to the inhibition of new CENP-A deposition until G1, when CDK1 activity is lost. However, non-phosphorylatable CENP-A (S68A) does not result in CENP-A loading outside of G1-phase; therefore, additional mechanisms further inhibit CENP-A deposition. There are conflicting data about the importance of Ser68 phosphorylation in cells. Fachinetti *et al.* [43] show no effect of a phosphomimetic mutant of Ser68 (S68D) on cell viability in gene replacement assays; whereas, Wang *et al.* [61] show that cell viability is reduced to 50% when the non-phosphorylated mutant (S68A) is introduced into the endogenous CENP-A locus by CRISPR. Ser18 within the amino terminal tail of CENP-A has also been proposed to negatively regulate CENP-A deposition; however, the mechanism is unknown [11].

Mis18BP1 and HJURP are the major targets of CDK phosphorylation in the CENP-A deposition pathway [51••,52••,59,62,63••] (Figure 3a,b). The expression of Mis18BP1 containing mutations within the CDK phosphorylation sites leads to CENP-A loading in G2; however, the level of CENP-A deposited is much lower than that observed in G1 [51••, 59,63••]. Phosphorylation of sites within the Mis18α/β binding domain in the amino terminus of Mis18BP1, specifically Thr40 and Ser110, inhibit the binding of the Mis18α/β heterohexamer [51••,52••] (Figure 3b), providing a molecular basis of the inhibition of CENP-A deposition by CDK1 phosphorylation of Mis18BP1. Phosphorylation of Thr653 also inhibits recruitment of the Mis18 complex to centromeres, but lies outside of the Mis18α/β interaction domain [63••], suggesting there is more to be learned about how phosphorylation by CDK1 inhibits this complex.

Likewise, the alanine mutations of CDK-dependent phosphorylation sites in HJURP (Ser210, Ser211 and Ser412) lead to HJURP recruitment to centromeres and loading of CENP-A in G2, but not to the levels observed when cells enter G1 (Figure 3b) [62,63••]. The mechanism of how HJURP phosphorylation inhibits recruitment and CENP-A deposition remains unclear. Regardless, combining phospho-mutants of HJURP and Mis18BP leads to accumulation of new CENP-A in G2 that is comparable to the level of CENP-A normally deposited in G1. These data suggest that the combinatorial modification of HJURP and Mis18BP1 by CDK1 accounts for the major regulation of CENP-A deposition. Although

mutations in HJURP and Mis18BP1 lead to unscheduled loading in G2-phase, S-phase seems to be refractory to new CENP-A deposition even under these conditions [62].

The PLK1 mediated phosphorylation of the human Mis18 complex also plays a regulatory role in new CENP-A deposition pathway. Inhibition of the PLK1 kinase activity results in new CENP-A deposition defects and mitotic errors [64]. The timing of CENP-A nucleosome deposition varies between organisms, and so the mechanisms that control CENP-A deposition are also likely to diverge from those discussed.

Influence of chromatin context on CENP-A deposition

Only a subset of the alpha-satellite DNA present at the centromere is occupied by CENP-A nucleosomes. Estimates based on *in vivo* fluorescence measurements suggest that there are approximately 200 CENP-A nucleosomes per centromere [65]. Histone H3 nucleosomes flank regions of CENP-A containing nucleosomes and are also interspersed between CENP-A nucleosomes within the endogenous centromere [66–68]. H3.3-variant nucleosomes predominate within the CENP-A domain [69]. The presence of H3.3 may reflect the active transcription of alpha-satellite DNA at the centromere [70]. Interspersed centromeric H3 nucleosomes are often marked by histone H3 Lys4 and Lys36 dimethylation [66,67]; while nucleosomes flanking the CENP-A nucleosomes contain histone H3 Lys9 dimethylation (Figure 4a). This suggests that the CENP-A containing alpha-satellite DNA are associated with both active and repressive marks. Although endogenous centromeres show H3 Lys4 and Lys36 methylation within the CENP-A domain, a strong correlation between centro-mere function and H3 Lys4 methylation has not been observed at neocentromeres, leaving open the question of whether these modifications are essential for centromere function outside of alpha-satellite repetitive regions [71].

The modification state of the histone H3 within chromatin is critical for the assembly of the centromere during G1. The efficiency with which centromeres form on exogenously provided alpha-satellite repeats of a human artificial chromosome (HAC) is influenced by the histone H3 Lys9 methylation/acetylation status. H3 Lys9 tri-methylation inhibits HAC stability and CENP-A accumulation on the alpha-satellite repeats of the HAC, and the presence of H3 Lys9 dimethylated histones limits the spread of CENP-A nucleosomes into flanking heterochromatin [72•,73] (Figure 4b). Notably, the removal of histone H3 Lys4 dimethylation by the artificial tethering of LSD1 (lysine-specific demethylase 1) also inhibits HJURP recruitment and new CENP-A deposition [67]. In contrast, the acetylation of H3 Lys9 within the alpha-satellite of the HAC, enhances new CENP-A deposition and HAC stability [72•]. The active alteration of H3 histone modification through the recruitment of histone modifying enzymes occurs to facilitate new CENP-A deposition. Mis18BP1 interacts with the KAT7 acetyltransferase complex and is necessary for new CENP-A deposition [74] (Figure 4b). Recruitment of KAT7 to the centromere antagonizes H3 Lys9 trimethylation, which leads to increased histone H3 turnover and enhanced new CENP-A deposition.

Conclusion

CENP-A overexpression and misincorporation at non-centromeric loci is observed in many cancers, and serves as a potent prognostic marker in conjunction with other centromere genes [75–78]. Inhibition of CENP-A amino terminal methylation and hyperphosphorylation of Ser18 by cyclin E1/CDK2 contributes to chromosome instability [11,12••], suggesting that perturbations in posttranslational modifications of CENP-A may contribute to cancer phenotypes. Although several E3 ligases have been identified that lead to degradation of non-centromeric CENP-A^{Cse4} in yeast, almost nothing is known about the pathways that mark and degrade non-centromeric CENP-A in humans. These processes may be at play in CENP-A overexpressing tumors and provide a potential way to target non-centromeric CENP-A for degradation.

The epigenetic specification of centromere location by the unique CENP-A containing nucleosome means that assembling new CENP-A nucleosomes as cells replicate their genome and divide, is a critical process to ensure the inheritance of the centromere. The recent studies reviewed here show that posttranslational modifications have emerged as important regulators of CENP-A deposition and function within the centromere.

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•• of outstanding interest

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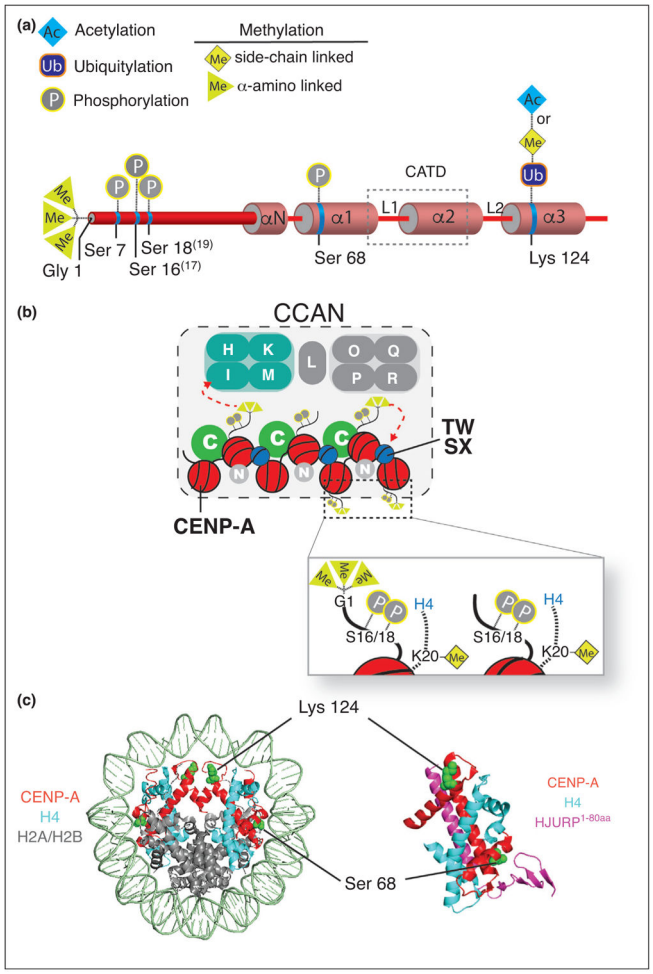

Figure 1.

Posttranslational modifications of CENP-A and CCAN recruitment. **(a)** A schematic of CENP-A secondary structure showing the locations of posttranslational modifications. Note that Ser16, Ser18 and Gly1 are numbered based on the removal of the initiating methionine, in convention with other histone proteins; however, these modifications have also been referred to by Ser17, Ser19 and Gly2, respectively. **(b)** Centromeric histone H4 and CENP-A posttranslational modifications found in centromeric chromatin. CCAN proteins (CENP-H/**I**/K/M, CENP-**T**/W/S/X and CENP-C) whose localization are influenced by CENP-A modifications are colored. **(c)** Location of phosphorylated Ser68 and modified Lys124 (green) within the CENP-A nucleosome (left) and the CENP-A/H4/HJURP^{Scm3} pre-nucleosomal complex (right). Based on crystal structures, 3AN2 (human CENP-A nucleosome) and 3R45 (human CENP-A and histone H4 heterodimer bound to HJURP amino acids 1–80).

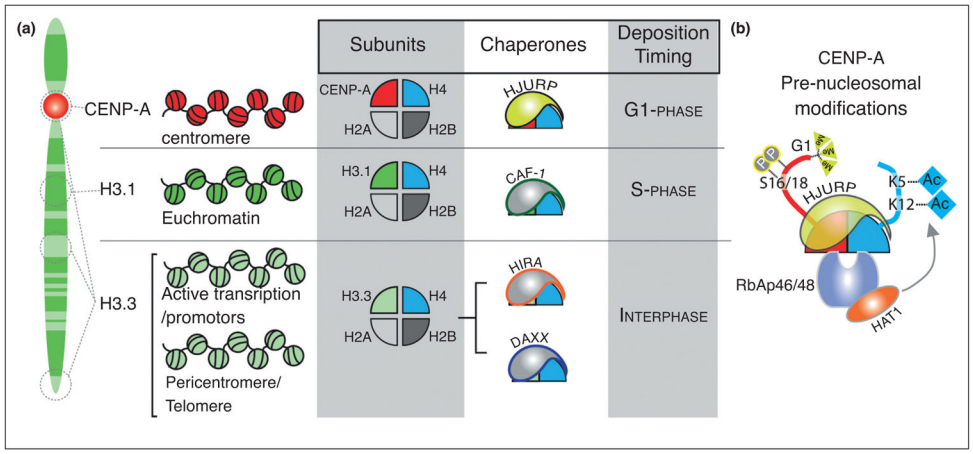

Figure 2.

Histone variant chaperones and pre-nucleosomal posttranslational modifications of CENP-A. **(a)** The interaction of histone H3 variants with distinct chaperone proteins determines the timing and site of variant nucleosome deposition. **(b)** The amino-terminus of prenucleosomal CENP-A is trimethylated and phosphorylated at Ser16/18. Recruitment of the HAT1 acetyltransferase through RbAp46/48 binding to CENP-A and histone H4 leads to acetylation of the histone H4 tail within the prenucleosomal complex.

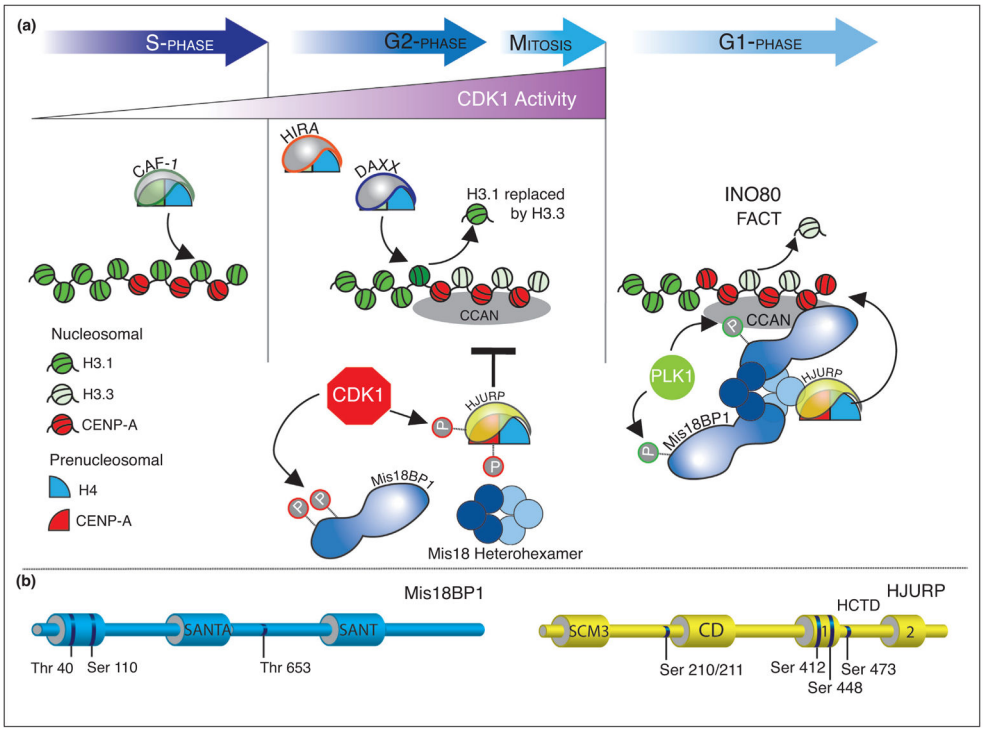

Figure 3.

Cell cycle control of vertebrate CENP-A deposition. **(a)** CDK1 activity increases in G2/M phase and inhibits the deposition of CENP-A nucleosomes by directly phosphorylating Mis18BP1 and the CENP-A chaperone HJURP. Mis18BP1 phosphorylation disrupts the binding to the Mis18α/β oligomer and thus inhibits recruitment of the complex to centromeres. Reduction of CDK1 activity in response to activation of the APC/C following mitotic checkpoint satisfaction leads to CENP-A deposition in G1. PLK1 activity positively regulates CENP-A deposition through phosphorylation of Mis18BP1. FACT mediated transcription in *Drosophila* and INO80 remodeling complex in budding yeast promote histone exchange within centromeric chromatin (reviewed by [70]). **(b)** CDK1 targeted phosphorylation sites within Mis18BP1 and HJURP that are known to inhibit CENP-A deposition are depicted.

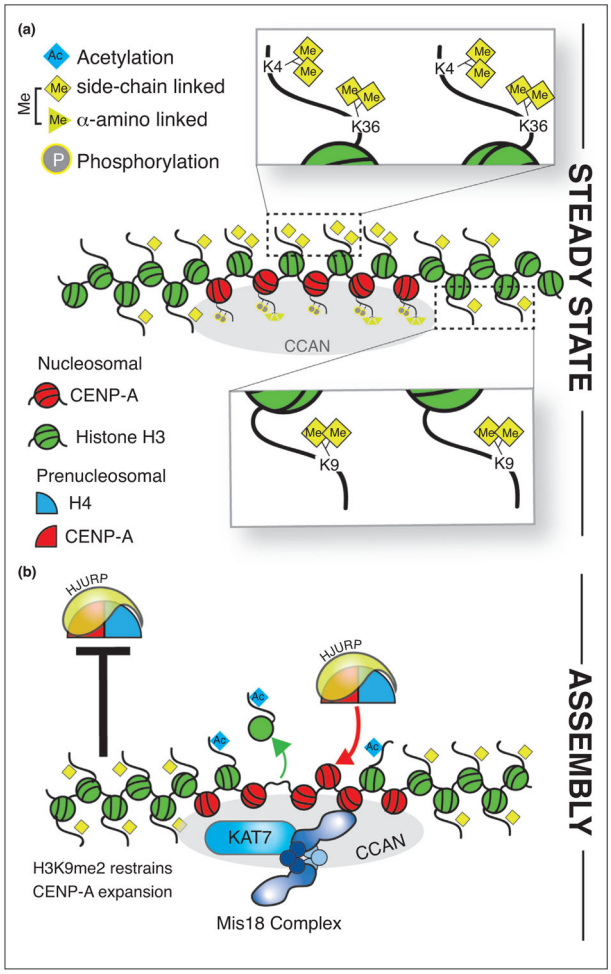

Figure 4.

Role of the chromatin context in CENP-A deposition. **(a)** Histone H3 nucleosomes are interspersed within and surround CENP-A containing chromatin throughout the cell cycle (steady-state). H3 nucleosomes within CENP-A containing chromatin are enriched for H3 Lys4 and Lys36 dimethylation, and H3 Lys9 dimethylation marks are found in nucleosomes flanking centromeres. **(b)** During G1-phase, recruitment of the KAT7 acetyltransferase complex by the Mis18 complex facilitates histone H3 removal and the assembly of new CENP-A nucleosomes.