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REVIEW

# Posttranslational modifications of CENP-A: marks of distinction

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## Abstract

Centromeres are specialized chromosome domain that serve as the site for kinetochore assembly and microtubule attachment during cell division, to ensure proper segregation of chromosomes. In higher eukaryotes, the identity of active centromeres is marked by the presence of CENP-A (centromeric protein-A), a histone H3 variant. CENP-A forms a centromere-specific nucleosome that acts as a foundation for centromere assembly and function. The posttranslational modification (PTM) of histone proteins is a major mechanism regulating the function of chromatin. While a few CENP-A site-specific modifications are shared with histone H3, the majority are specific to CENP-A-containing nucleosomes, indicating that modification of these residues contribute to centromere-specific function. CENP-A undergoes posttranslational modifications including phosphorylation, acetylation, methylation, and ubiquitylation. Work from many laboratories have uncovered the importance of these CENP-A modifications in its deposition at centromeres, protein stability, and recruitment of the CCAN (constitutive centromere-associated network). Here, we discuss the PTMs of CENP-A and their biological relevance.

**Keywords** CENP-A · Centromere · Posttranslational modification · Chromatin · Mitosis · Kinetochore

## Introduction

Equal distribution of chromosomes during mitosis is critical for normal cellular functioning and organism development. Errors in chromosome segregation can lead to a state of aneuploidy, which is defined by the presence of extra or fewer chromosomes than normal. Cancers are frequently aneuploid, and the specific loss or gain of tumor suppressor and oncogenes associated with changes in chromosome number may contribute to the prevalence of aneuploidy in cancer (Giam and Rancati 2015; Holland and Cleveland 2009; Pfau and Amon 2012; Sen 2000).

The centromere is a chromosomal locus that orchestrates chromosome movement during mitosis and meiosis. The kinetochore assembles at the site of the centromere and provides the physical attachment site between the chromosome and microtubule spindle. Likewise, the mitotic checkpoint signaling apparatus assembles at the centromere and delays mitosis until chromosome alignment is achieved (Cleveland et al. 2003; Fukagawa and Earnshaw 2014; Westhorpe and Straight 2014).

Although the centromere is classically viewed as the central constriction in mitotic chromosomes, the centromere persists throughout the cell cycle (Allshire and Karpen 2008). Centromeric DNA in humans ranges from 0.3 to 5 M base pairs and is comprised of higher order repeats (HOR) of 171 base pairs α-satellites (Fukagawa and Earnshaw 2014). While many organisms show this pattern of restricted centromere assembly embedded in unique repetitive DNA sequences, several organisms have distributed their centromeres across the entire length of the chromosome known as holocentromeres. (Drinnenberg et al. 2014; Neumann et al. 2015). Holocentromeres may exist in diffused pattern or may occupy several discrete sites throughout the chromosome (Melters et al. 2012).

The location of the centromeres in many yeasts and most-higher eukaryotes is determined epigenetically by the centromere protein-A (CENP-A), independent of DNA sequence (Black and Cleveland 2011; Choo 2000; Henikoff and Dalal 2005; Henikoff and Furuyama 2010; Perpelescu and Fukagawa 2011). At centromeres, CENP-A replaces the canonical histone H3 and forms nucleosome with H2A, H2B, and H4 (Black and Cleveland 2011). The centromere requires CENP-A for the recruitment of the constitutive centromere-associated network (CCAN) and kinetochore proteins in order

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to facilitate proper chromosome segregation (Carroll et al. 2010; Carroll et al. 2009; Cheeseman and Desai 2008; Fachinetti et al. 2013; Foltz et al. 2006; Hori et al. 2008; Izuta et al. 2006; Kato et al. 2013; Okada et al. 2006). Neocentromeres are the new centromeres that arise naturally and are independent of underlying DNA sequence and recruit CENP-A (Lo et al. 2001; Saffery et al. 2000; Warburton 2004). Assembling CENP-A containing chromatin at non-centromeric loci is sufficient to establish the centromere and assembles a kinetochore capable of microtubule attachments, demonstrating the primary role of CENP-A nucleosomes in forming the centromere (Barnhart et al. 2011; Bergmann et al. 2011; Guse et al. 2011; Mendiburo et al. 2011). Notably, CENP-A-containing nucleosomes are not arranged in a continuous fashion, but are instead interspersed with H3-containing nucleosome that contain a distinctive set of posttranslational modifications important for centromere function (Blower et al. 2002; Fukagawa 2017; Garcia Del Arco and Erhardt 2017).

Posttranslational modifications of centromeric chromatin components have emerged as an important regulator of overall structure and function of centromeres. Compared with canonical histones, which are subjected to a large combinatorial array of posttranslational modifications that directly or indirectly regulate their function, the degree and the information about CENP-A posttranslational modifications are more limited. Nonetheless, CENP-A undergoes a variety of posttranslational modifications including phosphorylation, ubiquitylation, methylation, and acetylation on its amino terminus and histone-fold domain and this review will summarize the known posttranslational modifications of CENP-A and their roles in the context of centromere biology.

## An overview of CENP-A structure and posttranslational modifications

The importance of CENP-A nucleosomes for centromere function and inheritance generates an obvious question: what special features does CENP-A possess that confer its centromere-specific function? Among H3 variants, CENP-A is the most diverse variant with just 48% overall similarity to canonical histone H3. It has a highly divergent N–terminus (Sullivan et al. 1994; Tachiwana et al. 2012), and a C–terminus-histone-fold domain (HFD), which is 62% identical to that of H3 (Fig. 1). Within the HFD, the loop1 (L1) along with flanking regions of α1 and α2 helices (aa 75-114) form the CENP-A targeting domain (CATD), which is necessary and sufficient for its centromere localization (Fig. 1) (Black et al. 2004). Sequences in the amino and carboxyl termini are not required for CENP-A deposition and appropriate targeting, but are involved in exerting CENP-A function in building the centromere (Fachinetti et al. 2013; Foltz et al. 2009; Logsdon et al. 2015). Likewise, while the CATD is important for targeting CENP-A to the centromere, the two amino acid "bulge" Arg-80 and Gly-81 in the structure contributes to CCAN recruitment (Sekulic et al. 2010; Tachiwana et al. 2011). Overall, differences in structural rigidity of the CENP-A nucleosome may also distinguish CENP-A from general chromatin and help serve its unique function (Black and Cleveland 2011; Black et al. 2010; Maddox et al. 2012; Stellfox et al. 2013).

Posttranslational modifications of canonical histone H3 regulate its function and affect a wide range of cellular processes, including cell differentiation, chromatin condensation, gene expression, and DNA replication and repair (Fig. 1) (Xu et al. 2014). The canonical histone H3 amino terminus is rich in lysine and arginine (especially at the N–terminus), which are frequently modified by acetylation and methylation. On the other hand the lysine content of CENP-A is extremely low compared with H3; therefore, the available sites of modification are fewer. And, although the N–terminus of CENP-A is also rich in arginine, these residues do not appear to be frequently modified, if at all. Thus, the degree to which CENP-A is posttranslationally modified is significantly less than H3. This suggests that in the case of lysine modification, including activating and repressing marks such as H3K4 and H3K27 methylation, CENP-A has evolved to be refractory to these types of control mechanisms (Bannister and Kouzarides 2011; Vakoc et al. 2006). Moreover, where H3 undergoes at least 17 different types of modifications (Xu et al. 2014), only four types of modifications, methylation, acetylation, phosphorylation, and ubiquitylation, have been identified for CENP-A (Fig. 1). Nevertheless, recent discoveries demonstrate the significance of CENP-A PTMs in centromere biology (Table 1). In the subsequent sections of this review, we will describe how these PTMs contribute to the function of CENP-A.

## Role of posttranslational modifications of CENP-A in mitosis and CCAN recruitment

Early work from Sullivan and colleagues showed that CENP-A is phosphorylated at Ser-7 (Zeitlin et al. 2001a). Canonical histone H3 is phosphorylated at Ser-10 by the aurora kinases, which is a hallmark of mitosis (Sawicka and Seiser 2012). It was hypothesized that CENP-A Ser-7 could also be subjected to phosphorylation by aurora kinases based on the similarly with histone H3 Ser-10. Despite their similarities, the temporal pattern of H3 and CENP-A phosphorylations appear to be slightly different during $G_2$/M phase of the cell cycle. H3 phosphorylation begins in early $G_2$ and persists throughout mitosis; whereas, CENP-A Ser-7 phosphorylation initiates in prophase, peaks during prometaphase and disappears in anaphase (Fig. 2a) (Zeitlin et al. 2001a). Both mitotic kinases Aurora-A and Aurora-B have been reported to phosphorylate CENP-A at Ser-7. While Aurora-A initiates CENP-

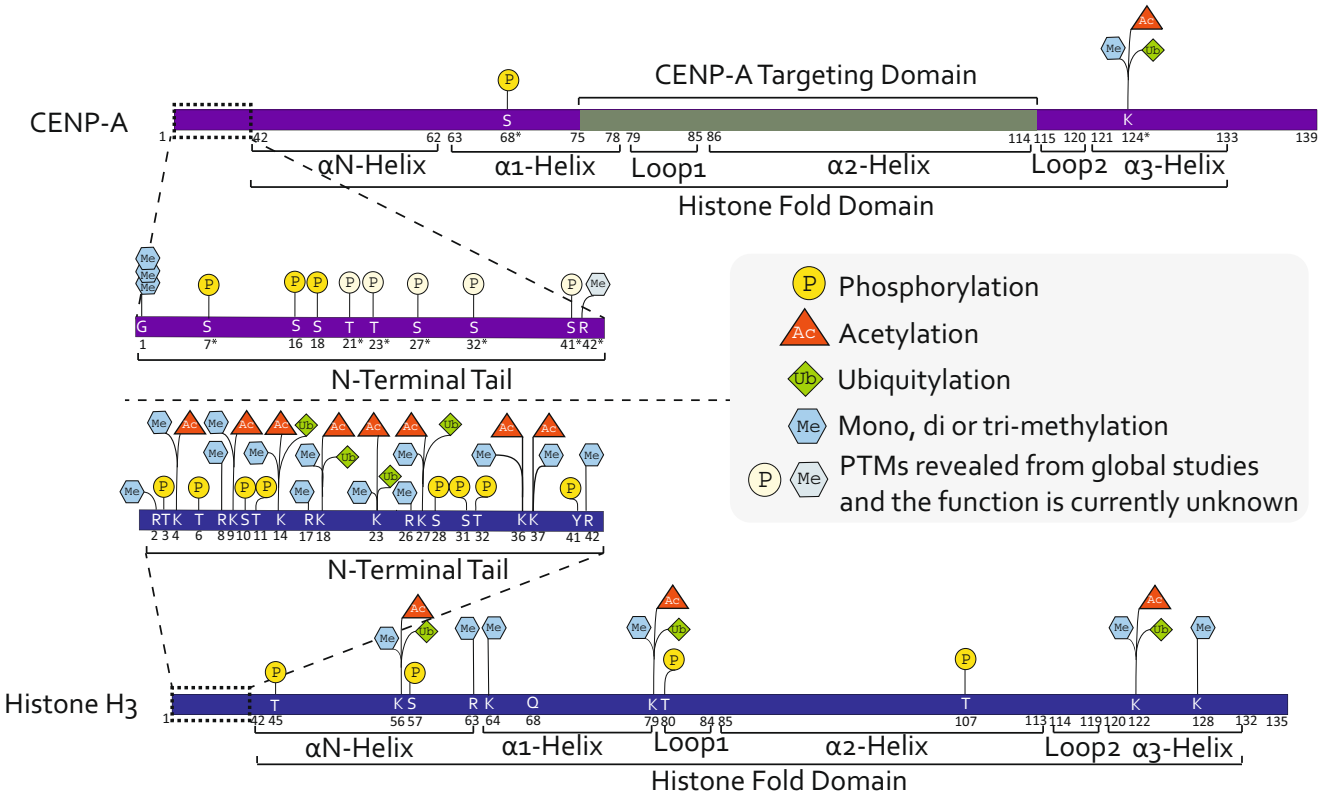


**Fig. 1** Posttranslational modifications of CENP-A and histone H3. A comparison of domains and posttranslational modifications of CENP-A and histone H3, which include methylation, phosphorylation, acetylation, and ubiquitylation. * In original articles, the initiating M cleavage was not taken into consideration and the position of these residues is shifted by + 1. The correct position of these residues is one unit less than depicted the diagram

A phosphorylation in prophase, Aurora-B is required for the maintenance of phosphorylation (Fig. 2a) (Kunitoku et al. 2003; Zeitlin et al. 2001b). Subsequent mass spectrometry-based studies failed to identify Ser-7 phosphorylation (Bailey et al. 2013) perhaps because Ser-7 phosphorylation is highly labile and was not significantly maintained in mass spectrometry-based experiments. Alternatively, only a small proportion of the CENP-A nucleosomes may be phosphorylated at Ser-7 during mitosis. Mass spectrometry analysis of yeast Cse4 revealed the phosphorylation of Ser-22, Ser-33, Ser-40, and Ser-105 (Boeckmann et al. 2013; Hoffmann et al. 2017) (Fig. 4). The phosphorylations at these four sites is mediated by Ipl1, the yeast homolog of Aurora-B. Such N–terminus phosphorylation of Cse4 by Ipl1/Aurora-B is reminiscent of human CENP-A Ser-7 phosphorylation and it has been shown that phosphorylation of Cse4 facilitates the destabilization of defective kinetochore to ensure the proper chromosome segregation (Boeckmann et al. 2013). Thus these data suggest the functional conservation of Aurora-B phosphorylation of CENP-A.

Studies performed using a human CENP-A phospho-defective mutant indicate the importance of Ser-7 phosphorylation in mitosis, but present contradictory results. Zeitlin et al. demonstrated that the prevention of Ser-7 phosphorylation does not affect kinetochore function, but leads to increased midbody persistence and size, and thus delays the completion of cytokinesis (Zeitlin et al. 2001b). On the other hand, Kunitoku et al. subsequently showed that non-phosphorylatable CENP-A results in misalignment of chromosomes and impaired kinetochore attachment to microtubules (Kunitoku et al. 2003). Although these studies differ in their conclusions regarding the specific function of Ser-7 phosphorylation, both the studies show Ser-7 phosphorylation to be important for Aurora-B localization at centromeres. In this way, CENP-A Ser-7 phosphorylation and Aurora-B appear to function bi-directionally, where Aurora-B maintains the Ser-7 phosphorylation initiated by Aurora-A and phospho-CENP-A in turn recruits Aurora-B to the inner centromere. Mutational analysis further substantiates the role of CENP-A Ser-7 phosphorylation in chromosome segregation and cytokinesis, as CENP-A phospho-defective mutants fail to rescue the mitotic defects caused by the loss of endogenous CENP-A (Goutte-Gattat et al. 2013).

Amino-terminal trimethylation of CENP-A, which was identified through high-resolution mass spectrometry, has emerged as a regulator of CENP-A function and is required for precise chromosome segregation during mitosis (Bailey et al. 2013; Sathyan et al. 2017). Similar to histone H3, the initiating methionine is constitutively removed from CENP-A and the enzyme NRMT1 trimethylates the alpha amino group of the exposed glycine residue on CENP-A (Fig. 2a). Notably, this modification is not observed on canonical H3. Although a

**Table 1** Human CENP-A Posttranslational Modifications

| Residue | Modification | Enzyme | Cell Cycle Stage | Function/Remarks | References |
|---|---|---|---|---|---|
| Gly-1 | Amino-terminal Trimethylation | NRMT1 | Constitutive but increased during mitosis | • Proper chromosome segregation<br>• CENP-I and CENP-T recruitment<br>• Prevention leads to multipolar spindle in p53-/- background | Bailey et al. 2013<br>Sathyan et al. 2017 |
| Ser-7 | Phosphorylation | Aurora A<br>Aurora B | Mitosis (Prophase and Metaphase) | • Mitotic progression and kinetochore function.<br>• Cytokinesis<br>• CENP-C localization through 14-3-3 | Zeitlin et al. 2001a<br>Zeitlin et al. 2001b<br>Kunitoku et al. 2003<br>Goutte-Gattat et al. 2013 |
| Ser-16<br>Ser-18 | Phosphorylation | Ser-16- Unknown<br>Ser-18-CDK2/ Cyclin E | Constitutive | • Ser-16 and -18 phosphorylations have been proposed to facilitate higher order chromatin organization<br>• Ser-18 phosphorylation inhibits CENP-A deposition and promotes tumor growth | Bailey et al. 2013<br>Takada et al. 2017 |
| Ser-68 | Phosphorylation | CDK1/Cyclin B | G2-phase/Mitosis | • Prevents CENP-A interaction with HJURP and deposition at centromeres | Hu et al. 2011<br>Yu et al. 2015<br>Wang et al. 2017 |
| Lys-124 | Ubiquitylation | CUL4A/RBX1/ COPS8 | Mitosis/G1-phase | • Facilitates CENP-A interaction with HJURP and deposition at centromeres | Niikura et al. 2015<br>Niikura et al. 2016<br>Niikura et al. 2017a<br>Niikura et al. 2017b |
| Lys-124 | Acetylation | p300 | G1-phase/S phase | • Inhibits CENP-C recruitment<br>• Precise function unknown but proposed to impact centromere dynamics | Bui et al. 2012<br>Bui et al. 2017 |
| Lys-124 | Monomethylation | Unknown | S-phase | • Precise function unknown | Bui et al. 2017 |

subset of CENP-A is methylated in randomly cycling cells, almost the entire pool of nucleosomal CENP-A is trimethylated by the time cells enter mitosis (Fig. 2a) (Bailey et al. 2013). Preventing CENP-A methylation in conjunction with loss of the p53 tumor suppressor leads to multipolar spindles. The CCAN protein CENP-T is reduced in cells expressing CENP-A that cannot be methylated on its amino terminus and reduced levels of CENP-T in these cells cause spindle multipolarity. Chromosome missegregation errors also occur when CENP-A cannot be methylated, but these errors are independent of p53 status. CENP-A methylation mutants leads to uncontrolled cell proliferation, and when expressed in *p53 −/−* null cells, results in early onset of tumors in nude mice, suggesting that misregulation of CENP-A methylation may have implications in cancer (Sathyan et al. 2017).

As a master component of the centromere, CENP-A recruits the CCAN, which is required for correct attachment of microtubules to the kinetochores and proper chromosome segregation during mitosis (Carroll et al. 2010; Carroll et al. 2009; Cheeseman and Desai 2008; Fachinetti et al. 2013; Foltz et al. 2006; Guse et al. 2011; Hori et al. 2008; Izuta et al. 2006; Kato et al. 2013; Okada et al. 2006). CENP-A phosphorylation, acetylation, and trimethylation appear to affect the recruitment of CCAN-associated proteins to the centromere. Previously, Ser-7 phosphorylation was found to be involved in loading of CENP-C onto centromeres through phospho-binding protein 14-3-3, which acts as an intermolecular bridge between phospho-CENP-A and CENP-C (Goutte-Gattat et al. 2013) (Fig. 2). More recently, both N– and C–termini of CENP-A have been shown to be independently involved in CENP-C recruitment to the centromere. The carboxyl tail facilitates direct recruitment of CENP-C, whereas the N–terminus recruits CENP-C via an interaction with CENP-B (Fachinetti et al. 2013; Fachinetti et al. 2015; Guse et al. 2011). Thus, the CENP-A Ser-7 phosphorylation may affect the loading of only a subset of CENP-C. Acetylation of CENP-A at Lys-124 has been suggested to impede the accessibility of CENP-C; however, it is not clear if this selectively affects N- or C-terminally associated CENP-C (Bui et al. 2017). Gly-1 trimethylation, on the other hand, regulates the localization of other components of CCAN, namely CENP-T and CENP-I without affecting CENP-C (Fig. 2b) (Sathyan et al. 2017).

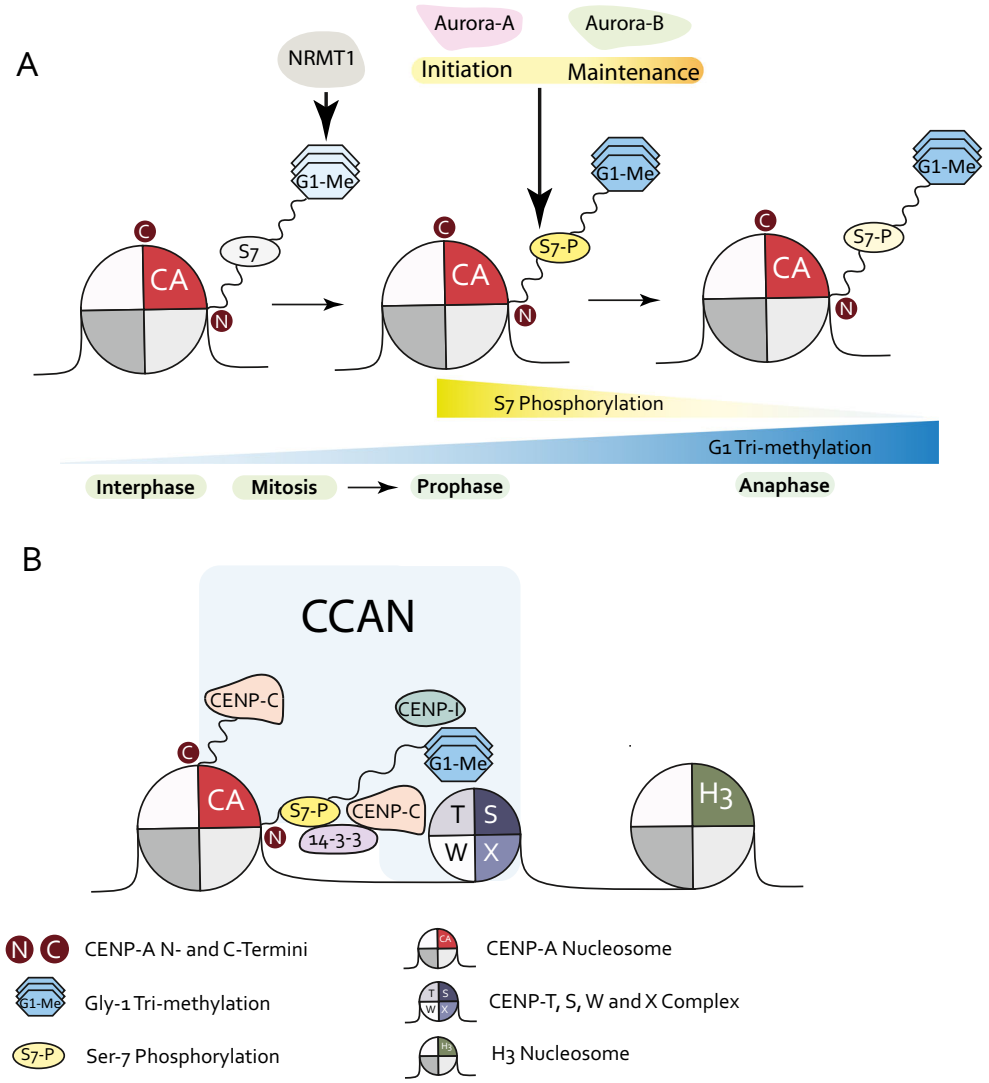


**Fig. 2** N-terminal modifications of CENP-A facilitate CCAN recruitment. **a** NRMT1 trimethylates CENP-A at Gly-1 in interphase; the proportion of which is increased during mitosis. Aurora-A initiates the Ser-7 phosphorylation in prophase, the mark is then maintained by Aurora B. During anaphase, Ser-7 phosphorylation is reduced. **b** CENP-A trimethylation facilitates the recruitment of CCAN components CENP-T and CENP-I. Ser-7 phosphorylation mediates the recruitment of CENP-C through 14-3-3 proteins. The CENP-A C-terminal tail also directly recruits CENP-C independent of Aurora phosphorylation

## CENP-A posttranslational modifications and deposition at centromeres

CENP-A nucleosome deposition occurs in $G_1$ phase and is directed by the chaperone HJURP (Bernad et al. 2011; Dunleavy et al. 2009; Foltz et al. 2009; Jansen et al. 2007; Shuaib et al. 2010). HJURP recognizes the CENP-A CATD (CENP-A targeting domain) through its N-terminal SCM3 homology domain and facilitates the loading of CENP-A-containing nucleosomes into DNA (Barnhart et al. 2011; Bassett et al. 2012; Dunleavy et al. 2009; Foltz et al. 2009; Jansen et al. 2007; Sanchez-Pulido et al. 2009; Shuaib et al. 2010). CENP-A deposition is regulated by the phosphorylation of its deposition machinery by two kinases, PLK1 and CDK1/2 (McKinley and Cheeseman 2014; Silva et al. 2012; Stankovic et al. 2017). While PLK1 is a positive regulator of CENP-A deposition, CDK1/2 is inhibitory. Both PLK1 and CDK are the regulators of CENP-A assembly factor Mis18 complex. The Mis18 complex, that includes Mis18BP1, Mis18α and Mis18β, is localized to centromeres during late telophase and early $G_1$ and is required for HJURP recruitment and new CENP-A deposition (Barnhart et al. 2011; Fujita et al. 2007; Hayashi et al. 2004; Nardi et al. 2016; Wang et al. 2014). PLK1 phosphorylates the Mis18 complex and promotes its centromere localization (McKinley and Cheeseman 2014). In contrast, CDK activity regulates multiple components of the deposition machinery to inhibit CENP-A deposition until mitosis is completed. Mis18BP1 is a substrate for CDK1 phosphorylation, and modification of the protein disrupts the association of the protein with the

Mis18α/β hexamer (Pan et al. 2017; Silva et al. 2012; Spiller et al. 2017). CDK1 also phosphorylates HJURP to inhibit CENP-A deposition (Muller et al. 2014; Stankovic et al. 2017). Thus the negative regulation by CDK1 ensures CENP-A deposition does not occur in $G_2$, but is restricted to early $G_1$, immediately following satisfaction of the mitotic checkpoint in human cells.

While the CENP-A CATD is sufficient to bind HJURP and directs CENP-A deposition to centromeres, the posttranslational modifications of two evolutionarily conserved residues, Ser-68 and Lys-124, that lie outside the CATD, have been demonstrated to affect CENP-A localization at the centromeres, although the functional aspects of these modifications remain a point of dispute and are discussed below (Hu et al. 2011; Niikura et al. 2015; Wang et al. 2017; Yu et al. 2015). More recently, another modification, Ser-18 phosphorylation that also exists outside CATD has been shown to negatively regulate CENP-A deposition (Takada et al. 2017). Modifications on histone H4 present in the prenucleosomal complex are also involved in CENP-A deposition. Histone H4 Lys-5 and Lys-12 acetylations occur in the pre-deposition complex, are removed after CENP-A nucleosome formation, and appear to be critical for deposition (Fukagawa 2017; Shang et al. 2016).

The crystal structure of the HJURP-CENP-A-H4 complex indicates that HJURP binds the CENP-A-H4 dimer, and this binding may be influenced by Ser-68 of CENP-A (Hu et al. 2011). In vivo, Ser-68 phosphorylation is governed by the opposing actions of the kinase CDK1 and phosphatase PP1α (Yu et al. 2015). Consistent with the relative activities of CDK1/PP1α, CENP-A Ser-68 phosphorylation occurs during $G_2$/M phase and as the cell cycle proceeds through mitosis and $G_1$, Ser-68 is dephosphorylated (Fig. 3).

Canonical histone H3 contains glutamine at the position comparable to CENP-A Ser-68, which suggests that phosphorylation of Ser-68 confers regulatory function unique to CENP-A. Substitution of Ser-68 with the bulky glutamine or phosphomimetic glutamic acid in CENP-A abolishes HJURP interaction and prevents its localization to centromeres, whereas the S68A mutant shows a robust binding with HJURP (Logsdon et al. 2015; Yu et al. 2015). The CATD in the context of the rest of histone H3 ($H3^{CATD}$) is sufficient to bind HJURP and direct its deposition to centromeres (Black et al. 2004; Black et al. 2007; Hu et al. 2011). CENP-A equivalent substitutions in this mutant ($H3^{CATD\text{-}Q68S}$) significantly enhances its ability to interact with HJURP (Logsdon et al. 2015; Yu et al. 2015). Nevertheless, the H3 mutant containing Ser-68 without the CATD remains unable to bind to HJURP, clearly reinforcing the idea that CATD is the primary determinant for CENP-A deposition to centromeres (Logsdon et al. 2015; Yu et al. 2015).

Similar to Ser-68, Ser-18 is also not essential for CENP-A deposition, but Ser-18 phosphorylation by Cyclin E/CDK2 complex appears to negatively regulate centromeric localization of CENP-A (Takada et al. 2017). Given that Cyclin E levels remain low during mitosis to $G_1$ transition, a hypophosphorylated state of Ser-18 may facilitate the specific timing of CENP-A deposition at centromeres in $G_1$ phase.

While phosphorylation of Ser-68 generates a potential steric hindrance and impairs CENP-A interaction with HJURP, this interaction is facilitated by Lys-124 ubiquitylation (Fig. 3) (Niikura et al. 2015). The SGT1-HSP90 complex facilitates the recognition of CENP-A by COPS8 and Lys-124 undergoes ubiquitylation by the CUL4A-RBX1-COPS8 complex (Niikura et al. 2016; Niikura et al. 2015; Niikura et al. 2017b) (Fig. 3). Fusion of ubiquitin to the C–terminus of CENP-A–K124R mutant restores the CENP-A interaction with HJURP and localization at centromere (Niikura et al. 2015).

In contrast to the dynamic nature of Ser-68 phosphorylation, so far, no deubiquitylating enzyme has been identified for Lys-124. However, Lys-124 has been proposed to possess different modifications at different times during cell cycle. Ubiquitylation, which takes place at mitotic exit and entry into $G_1$ phase switches to acetylation at $G_1$/S phase, which is further exchanged for monomethylation during S phase (Bui et al. 2012; Bui et al. 2017; Niikura et al. 2017a; Niikura et al. 2015). The interplay among E3 ligase, acetyl, and methyl transferases (and their opposing counterparts, if there are any for CENP-A) that determines the modification status of Lys-124 has not been mechanistically defined; however, these studies intimate that acetylation and monomethylation may check Lys-124 ubiquitylation until M/$G_1$ phase when CENP-A deposition occurs (Fig. 3).

Despite the biochemical evidence described above, it is unclear whether modifications of Ser-68 and Lys-124 are essential for CENP-A function. In order to address this question, Fachinetti and colleagues attempted to rescue *CENP-A* knockout RPE (retinal pigment epithelium) cells by expressing CENP-A modification mutants by viral transduction. In these experiments, individual Ser-68 phospho-mimetic mutants, or unmodifiable Lys-124 mutant, rescued cell viability resulting from loss of endogenous CENP-A (Fachinetti et al. 2017). Potential caveats of these experiments, including the degree to which mutant CENP-A overexpression may compensate for loss of CENP-A modifications have been proposed to support the importance of the Lys–124 and Ser-68 modifications at the cellular level (Niikura et al. 2017a; Wang et al. 2017). Indeed, Fachinetti and colleagues observed that LacI-fused CENP-A–S68Q mutant reduced HJURP recruitment at LacO array, which is in agreement with a negative role for Ser-68 phosphorylation in HJURP binding as reported by Guohong and colleagues. In contrast, these same experiments showed that the CENP-A–K124R mutant was as efficient as wild-type CENP-A in recruiting HJRUP to the LacO site (Fachinetti et al. 2017), casting additional doubt on whether K124 affects CENP-A deposition through the proposed model. The contradiction between the apparent biochemical impact of these mutations and the fact that they are dispensable for cell viability may suggest that while

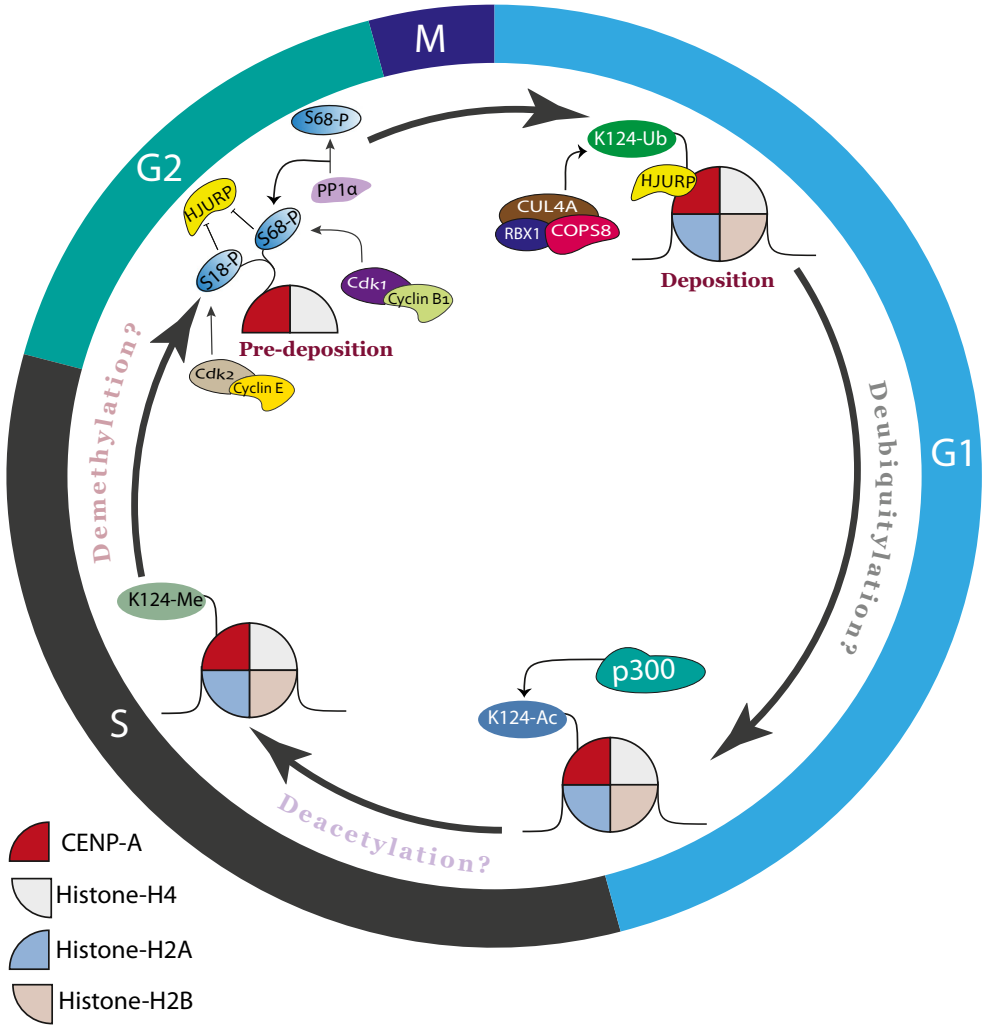


**Fig. 3** CENP-A posttranslational modifications during cell cycle. A diagram depicting modifications on CENP-A primarily involved in interaction with HJURP and centromere deposition. K124 undergoes multiple types of mutually exclusive modifications that include ubiquitylation, acetylation, and methylation. Phosphorylation of the CENP-A amino terminus at Ser-18 and Ser-68 are cell cycle regulated and may contribute to restricting the timing of HJURP binding

Ser-68 and Lys-124 are not absolutely required to regulate CENP-A recognition by HJURP, these modifications are likely to play a subtle modulatory role in CENP-A deposition than originally described. Further studies are warranted to resolve the remaining discrepancies.

## Diversity of CENP-A modifications across species

CENP-A shows a much higher degree of evolutionary divergence than its histone H3.1 counterpart, and these changes may be driven by co-evolution with the underlying centromeric DNA (Malik 2009; Rosin and Mellone 2017). The CENP-A N–terminus is the most variable region of the protein (Fig. 4). While the N-terminal sequence of CENP-A shows some conservation within the vertebrate lineage, more distantly related species are highly divergent (Fig. 4). Given the high degree of divergence, it is not surprising that the Ser-7 and Ser-16/18 modifications are only partially conserved in vertebrates, and mostly lost in other eukaryotes including flies, budding yeast, and nematodes. The histone-fold domains of CENP-A homologs are well conserved, as are the Ser-68 and Lys-124 modification sites within these domains. *Caenorhabditis elegans*, which does not contain an HJURP homolog, also does not show conservation at these sites. Interestingly, amino acid changes observed in zebrafish, flies, and fission yeast retain the charge at amino acid 124, but contain an arginine that would not undergo ubiquitylation or acetylation, suggesting that amino acid charge may be critical at this site (Fig. 4).

Consistent with the divergence of the CENP-A N–terminus, several modifications have been identified that are

unique to individual species. The *Drosophila* counterpart of human CENP-A (a.k.a. dCENP-A$^{CID}$) has been shown to be acetylated at Lys-105, located within the N–terminus, exclusively in cytosolic prenucleosomal fraction (Boltengagen et al. 2016). In addition, dCENP-A$^{CID}$ also undergoes phosphorylations at Ser-20, Ser-75, and Ser-77 (Boltengagen et al. 2016). The phosphorylation of dCENP-A$^{CID}$ Ser-75/77 is suggestive of human CENP-A Ser-16/18 phosphorylation; however, the functional consequences of these phosphorylation events appear to be different in *Drosophila* and human. While prenucleosomal cytosolic dCENP-A$^{CID}$ exhibits unphosphorylated, mono- or di-phosphorylated (Ser-20 and Ser-75) forms, the nucleoplasmic dCENP-A$^{CID}$ is enriched in an additional phosphorylation mark at Ser-77 (Boltengagen et al. 2016). Both acetylation and phosphorylation of *Drosophila* dCENP-A$^{CID}$ occur on the N–terminus, and based on their differential patterns in prenucleosomal and nucleosomal fractions, have been suggested to determine the localization of dCENP-A$^{CID}$ (Boltengagen et al. 2016).

dCENP-A$^{CID}$ has also been reported to be ubiquitylated, although the site of ubiquitylation is undetermined, and the effect of ubiquitylation is unique from human CENP-A. Ubiquitylation of dCENP-A$^{CID}$ is distinguished by the fact that the E3 ligase CUL3/RDX1 complex directly interacts with the functional homolog of HJURP in flies, called CAL1, which serves as an adaptor for the enzymatic reaction (Bade et al. 2014; Chen et al. 2014; Erhardt et al. 2008). CAL1 itself does not undergo ubiquitylation; nonetheless, both CAL1 and dCENP-A$^{CID}$ are stabilized by the CUL3/RDX complex, and loss of RDX leads to fragmented chromosomes and perturbs centromere maintenance.

The yeast homolog of human CENP-A, Cse4 is also posttranslationally modified. In addition to the functionally conserved Ipl1/Aurora-B sites found in the amino terminus of Cse4 (discussed earlier in the manuscript), the protein is methylated at Arg-37 and acetylated at Lys-49 (Boeckmann et al. 2013; Samel et al. 2012) (Fig. 4). While the function of Lys-49 acetylation is currently unknown, Arg-37 methylation is required for the recruitment of kinetochore components at centromeres, and its prevention leads to impaired chromosome segregation and growth defects in *Saccharomyces cerevisiae* (Samel et al. 2012).

CENP-A retention and its consistent levels at centromeres are crucial for genomic integrity. On the other hand, CENP-A overexpression is associated with its mislocalization outside the centromere to euchromatic regions in tumorigenesis (Allshire and Karpen 2008; Amato et al. 2009; Athwal et al. 2015; Filipescu et al. 2017; Hu et al. 2010; Lacoste et al. 2014; Li et al. 2011; Shrestha et al. 2017; Tomonaga et al. 2003; Wu et al. 2012; Zhang et al. 2016). Therefore, in normal

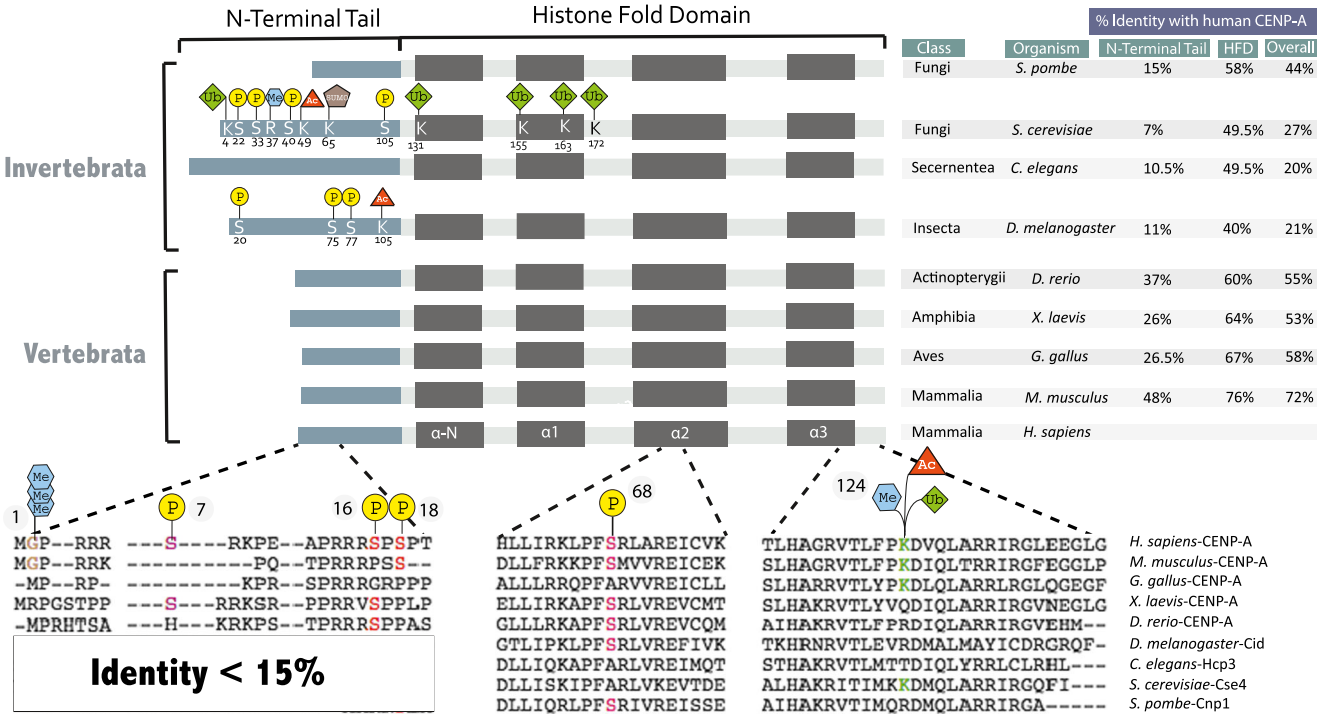


**Fig. 4** Conservation of human CENP-A posttranslational modifications across species. Schematic representation of CENP-A proteins in different organisms. Percent identity with respect to human CENP-A was computed through pairwise alignment using EMBOSS Needle program. CENP-A protein sequences in these organisms are also aligned through Clustal Omega and zoomed to focus on posttranslationally modified sites

conditions, there must exist a mechanism to eliminate CENP-A from non-centromeric loci. Ubiquitylation has been reported to prevent the ectopic localization of Cse4 by triggering its degradation, and the E3 ubiquitin ligases Psh1, Rcy1, Slx5, and Ubr1 have been shown to ubiquitylate Cse4 (Fig. 5) (Au et al. 2013; Cheng et al. 2017; Cheng et al. 2016; Collins et al. 2004; Hewawasam et al. 2010; Ohkuni et al. 2016; Ranjitkar et al. 2010). While Psh1 ubiquitylates Lys-4, Lys-131, Lys-155, Lys-163 and Lys-172 (Hewawasam et al. 2010), Slx5-mediated proteolysis of Cse4 is directed by sumoylation at Lys-65 (Ohkuni et al. 2018) (Fig. 4). The SUMO E3 ligases Siz1 and Siz2 target Cse4 for sumoylation, which further undergoes ubiquitylation by the ubiquitin ligase Slx5 (Fig. 5) (Ohkuni et al. 2016).

These E3 ubiquitin ligases function independently (Cheng et al. 2017). Despite Psh1 localization to centromeres, centromeric Cse4 is precluded from ubiquitylation through its interaction with the Scm3 chaperone (Hewawasam et al. 2010). Psh1 activity towards Cse4 is facilitated by casein kinase 2-mediated phosphorylation and its association with the chromatin modifying FACT (facilitates chromatin transcription/transactions) complex (Deyter and Biggins 2014; Hewawasam et al. 2014), providing additional levels of regulation.

Although human CENP-A is subjected to proteasomal degradation, the identity of the E3 ubiquitin ligases that act on it remains unknown (Earnshaw 2015; Lomonte et al. 2001). While Psh1 is not evolutionarily conserved, Rcy1, Slx5, and Ubr1 have human homologs, and loss of the human STUBL (SUMO-targeted ubiquitin ligase) ortholog RNF4 leads to chromosome segregation defects, suggesting the possibility that human CENP-A is regulated similar to Cse4 (van de Pasch et al. 2013). Moreover, the finding that Psh1 targets Cse4 through the functionally conserved CATD domain (Ranjitkar et al. 2010) supports the possibility for the existence of proteolytic regulation of human CENP-A akin to that which acts on Cse4. Taken together, the yeast studies have provided a tantalizing idea that changes in CENP-A stability may contribute to CENP-A overexpression and mislocalization in cancers, and it will be interesting to investigate whether this is the result of deregulation in the ubiquitin machinery targeting CENP-A.

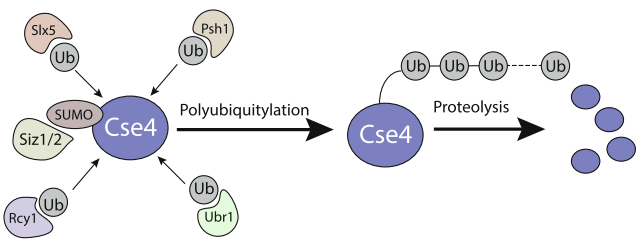


**Fig. 5** Cse4 ubiquitylation and proteolysis. Four E3 ligases Psh1, Rcy1, Ubr1, and Slx5 independently regulate Cse4 proteolysis through ubiquitylation. Note that ubiquitylation by Slx5 requires Siz1/2-directed sumoylation

## Perspective

Intrinsic or induced differences in the structure of the CENP-A nucleosome also contribute to distinguishing CENP-A chromatin from general chromatin (Falk et al. 2015; Sekulic et al. 2010). CENP-A posttranslational modifications that include Lys-124 acetylation and Ser-16/18 phosphorylation have the potential to influence the intrinsic properties of the CENP-A nucleosome or the CENP-A-containing chromatin (Bailey et al. 2013; Bui et al. 2017). CENP-A has been proposed to form a salt-bridged secondary structure through intra- and intermolecular association between phosphorylated Ser-16 and –18 and nearby arginine residues, both of which are highly conserved (Bailey et al. 2013). In vitro, Ser-16/18 salt bridging alters the organization of CENP-A-containing nucleosome arrays. In vivo, mutations that block CENP-A phosphorylation at these sites lead to chromosome missegregation, suggesting that organization of centromeric chromatin through the CENP-A amino-terminal tail may be important for centromere function. Lys-124 is located close to the pseudo-dyad DNA axis of the centromeric nucleosome and acetylation of this residue occurs during $G_1$/S phase of the cell cycle (Bui et al. 2017). Based on the similarity with canonical H3 K122 acetylation and molecular dynamic simulations, it has been proposed that acetylation of Lys-124 tightens the CENP-A nucleosome, potentially contributing to its unique function. However, clear experimental data is not yet available to substantiate the structural changes induced by Lys-124 modification.

CENP-A PTMs regulate CENP-A deposition at centromeres, protein stability, and CCAN recruitment, and thus, these PTMs play an important role in faithful segregation of chromosomes during cell division. Growing evidence suggests the potential alteration of CENP-A PTMs associated with cancer progression. The observation that a CENP-A methylation defective mutant together with loss of *p53*, promotes tumor development in nude mice and the correlation of Ser-18 hyperphophorylation with cyclin E-driven tumors, indicate a potential role of CENP-A modifications in cancer (Sathyan et al. 2017; Takada et al. 2017). Large-scale proteomic studies have revealed several additional modifications; the biological relevance of which is yet to be discovered (please see http://www.phosphositeplus.com). Clearly, our current knowledge of CENP-A PTMs is just the tip of the iceberg and offers enormous possibility of future research.

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