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Canonical and noncanonical regulators of centromere assembly and maintenance

Catalina Salinas-Luypaert and Daniele Fachinetti

Abstract

Centromeres are specialized chromosomal domains where the kinetochores assemble during cell division to ensure accurate transmission of the genetic information to the two daughter cells. The centromeric function is evolutionary conserved and, in most organisms, centromeres are epigenetically defined by a unique chromatin containing the histone H3 variant CENP-A. The canonical regulators of CENP-A assembly and maintenance are well-known, yet some of the molecular mechanisms regulating this complex process have only recently been unveiled. We review the most recent advances on the topic, including the emergence of new and unexpected factors that favor and regulate CENP-A assembly and/or maintenance.

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Introduction

Maintenance of genome integrity and accurate transmission of the genetic information at each cell division are paramount for the healthy development and survival of all living organisms. The segregation of eukaryotic chromosomes is mediated by the centromere, a specialized chromosomal domain that defines the locus for the assembly of the kinetochore. Proper centromere identification and function are essential for correct kinetochore assembly and chromosome attachment to the spindle microtubules, thus preventing the occurrence of aneuploidy, a hallmark of cancer [1]. While centromeric function is evolutionary conserved, centromere specification, structure and composition are diverse [2].

Humans and many other eukaryotic species are monocentric, having a single spatially delimited centromere per chromosome. Ensuring the maintenance of a unique centromere is critical in monocentric organisms since the loss of the centromere or gain of an additional one causes defects in chromosome segregation [3].

In most plants and animals, the centromeres assemble on tandem repeats of satellite DNA sequences that create complex repetitive arrays up to 5 mega-bases long in human and that have an accelerated rate of single-nucleotide and structural variations [4,5]. The existence of neocentromeres deprived of canonical repetitive sequences [3] demonstrate that centromeric DNA is, however, not necessary to sustain centromeric function. In most organisms, centromeres are epigenetically defined by a unique chromatin containing the histone H3 variant CENP-A (Centromere Protein A) [6], even though CENP-A is not universally required for centromere specification [1].

CENP-A nucleates the assembly of the constitutive centromere-associated network (CCAN), composed in vertebrates of sixteen centromeric proteins (CENPs) that stably associate with the centromeric chromatin throughout the cell cycle and form the inner kinetochore network during mitosis [7]. Recent structural models of the budding yeast [8] and human CCAN associated with the centromeric DNA [9–11] and a CENP-A nucleosome [9] have provided the most complete insights into the intricate molecular organization of this large proteinaceous complex. These models have also raised important questions regarding the relative importance of the CENP-A nucleosome and the CCAN components during mitosis [12,13], reinforcing similar concepts raised from previous work in human cells [14,15]. While CENP-A is key to define and assemble a functional centromere in most species [16], it does not actively participate in the chromosome segregation process, a task that falls fully on components of the CCAN. This notion is also supported by the fact that organisms that do not have CENP-A orthologues still depend on CCAN orthologues for chromosome segregation [17]. This review provides a contemporary view on well-established and novel regulators of centromere assembly and maintenance, with a focus on CENP-A deposition in human.

The canonical pathways for centromere maintenance

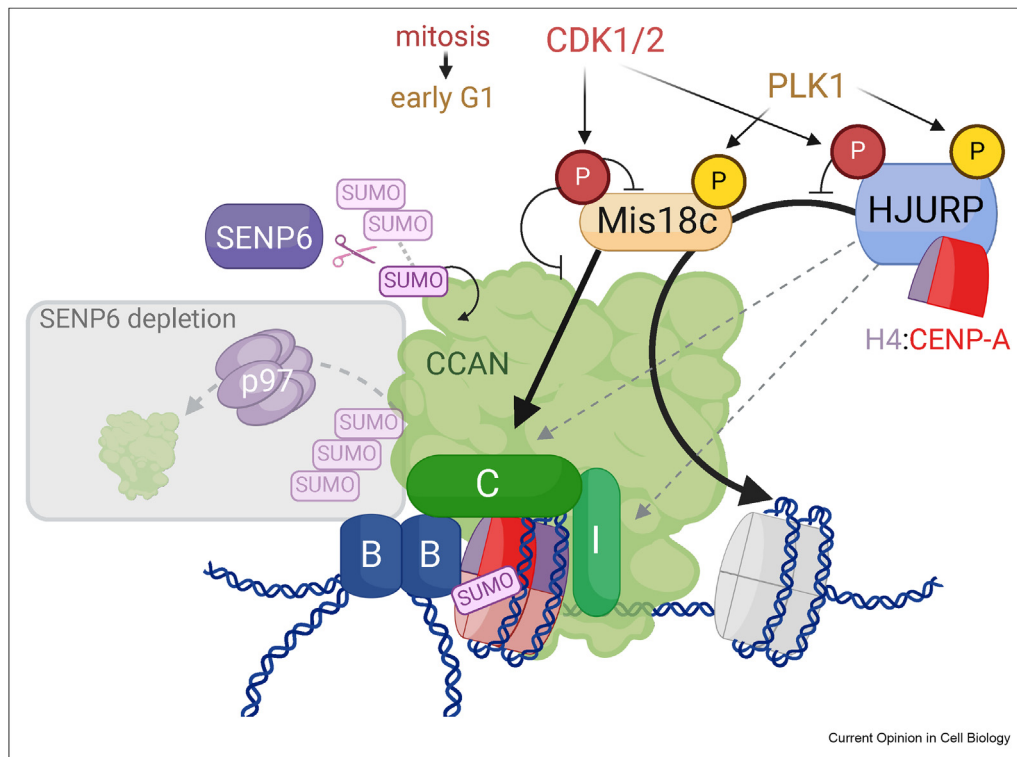
The deposition of CENP-A nucleosomes at the centromeres is cell-cycle-regulated and occurs during late telophase to early G1 [18,19], uncoupled from DNA replication. Preexisting CENP-A molecules promote a self-assembly epigenetic loop that allows centromeres to propagate accurately through cell divisions [20]. However, the presence of previously deposited CENP-A is not strictly necessary for its de novo deposition, as in the absence of CENP-A this function can be fulfilled by CENP-C and CENP-B [15].

The cornerstone players involved in CENP-A deposition include CCAN components as readers of preexisting CENP-A molecules, the reloading licensing Mis18 complex (Mis18C), and the CENP-A chaperone Holiday Junction Recognition Protein (HJURP), all controlled by posttranslational modifications [21] (Figure 1).

Readers of CENP-A, the mediators of the self-assembly epigenetic loop

Among all CCAN components, only CENP-C and CENP-N have known CENP-A binding domains. The N-terminal domain of CENP-N specifically binds CENP-A nucleosomes [22], although the recent cryo-EM structures of the CCAN indicate a steric clash between CENP-A and CENP-N [9–11], leading to the idea of a maturing CCAN structure that may be remodeled after its initial assembly [13]. CENP-C interacts directly with the C-terminal tail of CENP-A [23–25] via its central domain [23] and CENP-C motif [25,26], and is known to play a fundamental role in the incorporation and stabilization of CENP-A nucleosomes [27–29]. Recently it has been reported that the self-oligomerization of human and chicken CENP-C through the cupin domain is key for mediating CENP-C functions [30]. CENP-C provides a direct link between the existing CENP-A centromeric chromatin, members of the CENP-A reloading licensing Mis18C [27,31], and

Figure 1



Canonical regulators of centromere assembly and maintenance. The centromeric chromatin is represented with a nucleosome containing CENP-A (in red) upon which the CCAN is assembled (CENP-C and CENP-I are highlighted), with a canonical H3-containing nucleosome (in grey) and with a DNA-loop formed through the dimerization of CENP-B bound to the centromeric DNA. The well-established self-assembly loop of CENP-A is presented in bold arrows: the Mis18C is recruited to the centromere through its direct interaction with CENP-C and subsequently HJURP is brought to the centromere to deposit a new H4:CENP-A dimer by interacting with the Mis18C. Dashed arrows represent the proposed alternative routes of direct HJURP recruitment by interacting with CENP-C and CENP-I. During mitosis, high CDK1/2 activity phosphorylates HJURP and components of the Mis18C, limiting both the assembly of the complex and its recruitment to the centromeres. In early G1, the drop in CDK1/2 relieves these inhibitory phosphorylations and new PLK1-dependent phosphorylations promote the centromeric recruitment of both Mis18C and HJURP. The positive monoSUMOylations of the CCAN and CENP-A are depicted and are maintained by the action of the deSUMOylase SENP6. In its absence, polySUMOylation of the CCAN (and CENP-A, not depicted) leads to their p97-dependent extraction from the centromeres. Created with [BioRender.com](https://www.biorender.com).

CENP-A's chaperone HJURP [32,33] (Figure 1). It has been recently described that in the context of artificial kinetochore formation in chicken DT-40 cells, CENP-C interacts directly with HJURP, and that both CENP-C and CENP-I, another protein of the CCAN, recruit CENP-A independently of the Mis18C [34]. CENP-C was also shown to interact with CENP-B [35,36,14], the only centromeric protein that binds specifically to a conserved motif in the centromeric DNA [37]. CENP-B directly reshapes centromeric chromatin by promoting the formation of DNA loops [38] and by inducing nucleosomal DNA unwrapping and opening of the chromatin [39] (Figure 1). CENP-B/CENP-C interaction tethers the kinetochore to the centromere independently of CENP-A [14] and provides an alternative landmark for the maintenance of centromere position by promoting first CENP-C and then CENP-A deposition [15]. Interestingly, it was recently shown that CENP-I also directly interacts with the centromeric DNA, and that this interaction is required for the centromeric loading of newly synthesized CENP-A and the posterior stabilization of deposited CENP-A nucleosomes [40].

The Mis18 complex, CENP-A's reloading licensing factor

The highly conserved Mis18C is composed of four Mis18 α , two Mis18 β and two Mis18 Binding Protein 1 (M18BP1) subunits [41]. The complex is recruited to the centromeres in anaphase through a dual CENP-C recognition by Mis18 β and M18BP1 [31] and is crucial to prime the centromeres for new CENP-A deposition [42], mediating the recruitment of HJURP [41,43] (Figure 1). Mis18 β and the N-terminal tail of M18BP1 directly interact with the C-terminus of CENP-C, specifically targeting the complex to the centromeres [31]. Mis18 α also interacts with the N-terminus of M18BP1, and this domain is both necessary and sufficient for centromeric localization of the whole complex [31]. In non-mammalian vertebrates such as *Xenopus laevis* [33] and chicken [44] M18BP1 has been described to directly bind to CENP-A nucleosomes through its CENP-C-like motif simultaneously recognizing CENP-A's characteristic C-terminal tail and RG-loop [45]. The assembly and recruitment of the Mis18C in humans is cell-cycle-regulated by the cyclin-dependent kinases (CDK1/2) [46] and polo-like kinase 1 (PLK1) [47] (Figure 1). CDK-dependent phosphorylation of M18BP1 in Thr40 and Ser110 residues inhibits the assembly of the complex [41], while phosphorylation of Thr653 blocks the centromeric localization of M18BP1 without affecting its interactions with Mis18 α and Mis18 β [48]. The drop in CDK activity at the end of mitosis relieves these inhibitory phosphorylations and, concomitantly, the phosphorylation of the N-terminus of M18BP1 in early G1 by PLK1 [47], promotes the recruitment of the complex to the centromeres. Two recent preprints have provided new insights into PLK1's

cascade, revealing that it is first recruited to the centromeres by phosphorylating and engaging with two M18BP1 residues, Thr78 and Ser93 [49,50]. Subsequently, PLK1 phosphorylates and engages with Mis18 α Ser54, relieving the steric blockade of Mis18 α 's N-terminal region on the HJURP binding site, therefore positively regulating the recruitment of HJURP and allowing CENP-A deposition. These opposing and cell-cycle regulated phosphorylations act in a concerted manner likely to prevent noncentromeric CENP-A deposition while at the same time limiting its centromeric deposition strictly to late M/early G1. M18BP1, Mis18 α and several components of the CCAN have been also reported to be SUMOylated throughout the cell cycle [51–53]. While monoSUMOylation seems to be important for the stability of the CCAN and CENP-A chromatin, polySUMOylation leads to the recruitment of the ATPase p97, which acts as a protein segregase, extracting CCAN proteins and CENP-A nucleosomes from the centromeres [54]. p97 action is prevented by the deSUMOylating activity of the SUMO specific peptidase 6 (SENP6) and in its absence most CCAN proteins as well as CENP-A are lost from the centromeres [52]. A delicate SUMOylation balance seems therefore to play an important role in maintaining centromere homeostasis [55] (Figure 1). While the precise function of these posttranslational modifications is unknown, we hypothesize that they might serve as a switch mechanism, allowing to circumscribe centromere functionality within defined boundaries and/or to turn off this function in cases where it is no longer required. A physiological example of the latter hypothesis can be found in terminally differentiated muscle cells, which are known to lose CENP-A nucleosomes [56].

HJURP, CENP-A's chaperone

CENP-A assembly is mediated by its chaperone, HJURP [57,58]. Human HJURP forms a complex with the prenucleosomal CENP-A:H4 dimer, recognizing the CENP-A Targeting Domain (CATD) on CENP-A [58], and requiring either a sequential deposition of two dimers by the same HJURP molecule or a coordinated deposition of two dimers by two HJURP molecules to load a CENP-A:H4 tetramer in a single nucleosome [59] (Figure 1). The Mis18C recruits HJURP [60,61] through the interaction of the C-terminal domains of HJURP with three helix bundles from the C-terminus of two Mis18 α and Mis18 β subunits [59]. Alternatively, HJURP was proposed to be recruited to the centromeres by interacting with CENP-C [32,33]. The fungal orthologue of HJURP, Smc3, was also shown to interact directly with the centromeric DNA through a cysteine-rich domain [62]. As for the Mis18C, HJURP's localization to the centromere is regulated by a CDK-dependent phosphorylation on Ser210/211 and Ser412, which prevents its untimely recruitment [20,61,48] (Figure 1). It was recently determined that the mitotic

phosphorylation of *X. laevis* HJURP on the conserved Ser220 residue specifically prevents its interaction with the cupin dimerization domain of CENP-C in metaphase, blocking CENP-A deposition [63].

Emerging, noncanonical regulators of centromere assembly and maintenance

Unexpected factors have recently emerged, favoring and regulating CENP-A assembly and/or maintenance. Below is a recent overview of these noncanonical regulators.

DNA damage-related factors

New lines of evidence suggest that DNA damage-related proteins play a role in the assembly and maintenance of the centromeric protein network, likely due to an enrichment of these factors at centromeres [64]. Recent reports demonstrate high levels of intrinsic DNA breaks within human centromeres associated with DNA replication [65] and, also, in nondividing cells [66]. This fragility likely derives from the highly repetitive nature of the centromeric DNA and these centromeric breaks have been widely associated with genome instability [67]. As there is an enhanced need of repair at this genomic locus it is possible that DNA repair and the epigenetic maintenance of centromere identity (i.e. CENP-A deposition) evolved to be coupled together. In this regard, it was shown in quiescent cells that the depletion of the DNA recombinase RAD51, one of the key catalyzers of the homologous recombination DNA repair pathway in humans, increased the frequency of double-strand breaks within the centromeres and caused a striking reduction of CENP-A levels [66]. In this context, the authors suggest that RAD51 has a role in protecting the centromeric DNA from excessive DNA damage and propose a model where centromeric DNA breaks, and the subsequent RAD51-dependent recombination, promote the deposition of CENP-A (Figure 2a). The exact molecular mechanism of how RAD51 promotes CENP-A deposition remains to be addressed. The level of DNA breaks was also seen to correlate with CENP-A chromatin occupancy across centromeres in dividing cells [66], supporting previous controversial reports of CENP-A recruitment to break sites [68].

The ataxia telangiectasia and Rad3-related kinase (ATR), a key regulator of DNA damage and replication stress response, was first described to localize at mitotic centromeres by interacting with CENP-F, engaging with centromeric R-loops and stimulating Aurora B kinase activity to prevent the formation of lagging chromosomes [69]. More recently, ATR has been implicated in the protection of centromeric CENP-A in a DNA damage-independent manner by promoting DAXX (the chaperone of histone H3.3) association with the promyelocytic leukemia nuclear bodies (PML NBs) through its phosphorylation at Ser707 and Ser712, hence

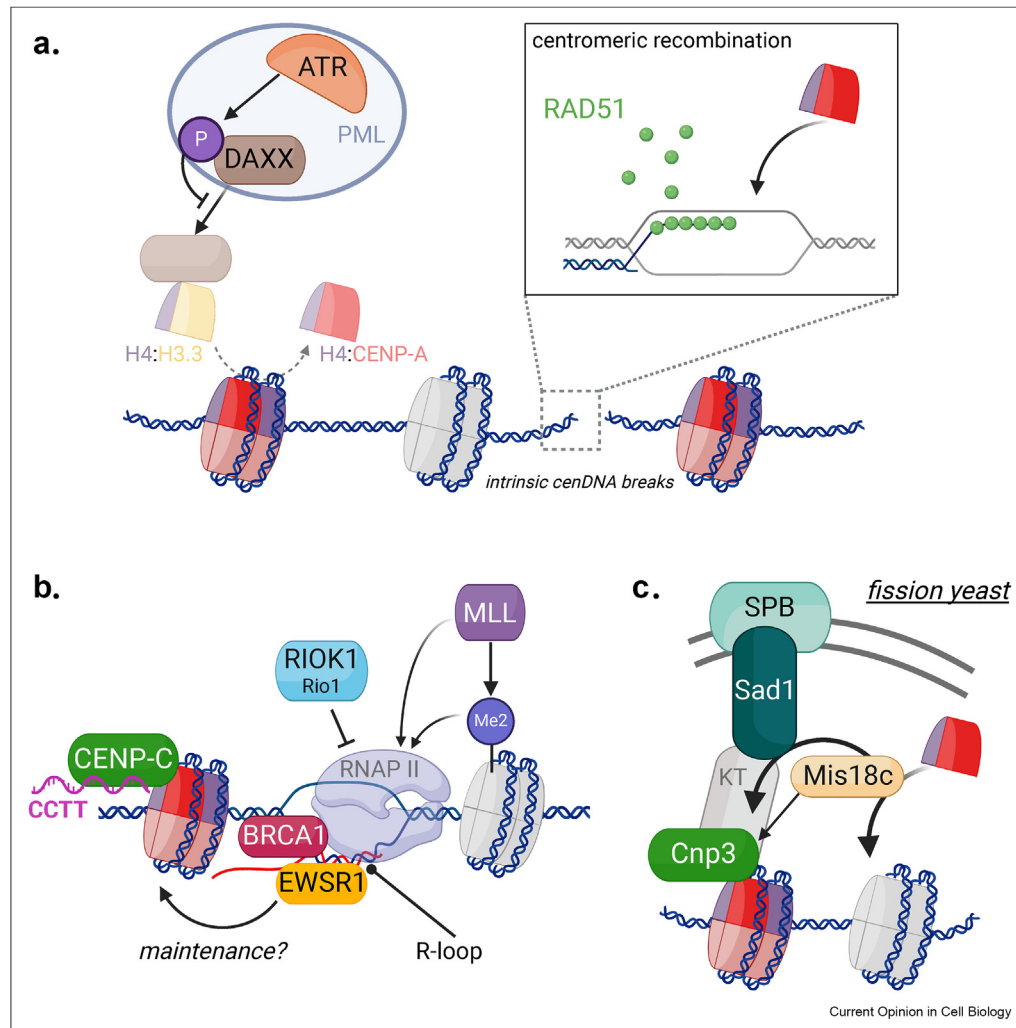
limiting aberrant H3.3 deposition at the centromeres [70] (Figure 2a). Inhibition of ATR in interphase led to a mild CENP-A eviction and DAXX-dependent H3.3 deposition at the centromeres, which resulted in chromosome segregation defects during mitosis. What led to these mitotic defects remains to be determined, as faithful chromosome segregation can occur even with low levels of CENP-A [24,71].

Centromeric transcription and transcripts

Centromeres are known to be transcribed and centromeric transcripts and transcription *per se* have been widely implicated in centromere maintenance and function [72]. A clear and definitive mechanism linking active centromeric transcription and CENP-A deposition has yet to be defined. It is likely that centromeric transcription could be a driver of nucleosome turnover, thereby facilitating the access of CENP-A's deposition machinery to the centromeres. The histone H3 dimethylation on lysine 4 (H3K4me2) is a well-known modification that promotes centromeric transcription and a recent study has revealed that the mixed lineage leukemia protein 1 (MLL1) and the SET Domain Containing 1A (SETD1A) lysine methyltransferases localize to the centromeres throughout the cell cycle to deposit this mark [73] (Figure 2b). CRISPR-mediated downregulation of MLL1 causes loss of H3K4me2, altering the epigenetic chromatin state of the centromeres with concomitant reduction of acetylation of H3K9 and an increase in H3K9me3. Downregulation of both methyltransferases reduced the centromeric levels of CENP-B and CENP-C and led to a decreased recruitment of HJURP and CENP-A in G1. These enzymes were also seen to promote centromeric transcription through their transcription activation domains and loss of MLL promoted R-loop formation by increasing the levels of elongating RNA polymerase II [73] (Figure 2b).

R-loops, three-stranded structures comprised of a DNA:RNA hybrid and a single-stranded DNA, are known to be enriched at centromeres [64,72,74,75]. If these structures play a role in centromere function or they simply accumulate there due to the repetitive nature of centromeres is still unclear. To the best of our knowledge, no evidence indicates that there is enrichment of R-loops at neocentromeres, which are formed in nonrepetitive DNA. Most probably they participate in the maintenance of the topology of the centromere, favoring the recruitment of additional factors that are important for chromosomal segregation. Recent works point in this direction, as a direct association between CENP-A and R-loops was demonstrated *in vitro* [76]. The authors propose a role for the RNA-binding protein Ewing sarcoma breakpoint region 1 (EWSR1) in anchoring CENP-A to the centromere through its binding of the centromeric transcripts (Figure 2b).

Figure 2



Emerging regulators of CENP-A deposition and maintenance a. DNA damage-related factors. (Left) ATR phosphorylation promotes DAXX association with promyelocytic leukemia (PML) nuclear bodies, effectively sequestering the histone H3.3 chaperone away from the centromeres and blocking the DAXX-dependent H3.3 deposition and consequent CENP-A eviction. (Right) The intrinsic centromeric DNA (cenDNA) fragility causes double-strand breaks both in replicating and quiescent cells. RAD51 protects the cenDNA from excessive DNA damage and RAD51-mediated recombination promotes the deposition of CENP-A. b. Centromeric transcription and transcripts. The centromeric chromatin is depicted as being transcribed by RNA polymerase II (RNAP II), forming a centromeric R-loop that is bound by BRCA1 and Ewing sarcoma breakpoint region 1 (EWSR1) proteins, which are proposed to maintain centromeric CENP-A levels. *S. cerevisiae* Rio1 and its human orthologue RIOK1 limit the centromeric transcription by reducing RNAPII accessibility, hence maintaining CENP-A levels. Members of the mixed lineage leukemia (MLL) lysine methyltransferase family deposit the H3K4me2 mark that directly promotes centromeric transcription and favors maintenance and the recruitment of several centromeric components. c. Lamin A-associated proteins and the LINC complex. In fission yeast, the centromeres associate with the spindle pole bodies (SPB), which are stable throughout the cell cycle. Sad1, the major SPB linker of the nucleoskeleton and cytoskeleton (LINC) component, recruits the Mis18C independently of CENP-C^{Cnp3}, therefore concentrating the Mis18C near the centromeres and favoring CENP-A^{Cnp1} deposition. Created with [BioRender.com](https://www.biorender.com).

Conditional degradation of EWSR1 led to CENP-A loss [76]; however, the exact molecular mechanism of this phenomenon remains to be determined. BRCA1, a tumor suppressor involved in many processes required to ensure chromosomal stability, could also be indirectly implicated in CENP-A localization via R-loop regulation. Indeed, BRCA1 has been seen to associate with R-loops at the centromeres to counteract their accumulation and BRCA1-deficient cells show impaired CENP-A localization, higher centromeric transcription, increased

centromeric breaks, and a RAD52-dependent hyper-recombination [77] (Figure 2b).

Through an unbiased approach, Zhang and collaborators have identified a previously unannotated long noncoding RNA transcribed from an arm of chromosome 17 which they renamed CENP-C targeting transcript (CCTT) [78]. The authors reported that CCTT binds specifically to CENP-C throughout the cell cycle via RNA-protein interactions and to the centromeric DNA,

possibly forming a RNA-DNA triplex (Figure 2b). The depletion of CCTT markedly reduced overall CENP-C levels, particularly new CENP-C deposition at the centromeres, and consequently led to mitotic errors and chromosomal instability.

Another factor at the interplay between centromeric transcription and CENP-A assembly was recently proposed in both budding yeast and human cells. The *Saccharomyces cerevisiae* protein kinase/ATPase Rio1, known to downregulate RNA polymerase I (RNAPI) activity and promote the prerRNA processing, was described to limit the centromeric transcription by reducing RNAPII accessibility and promoting the transcript's degradation by the 5'–3' exoribonuclease Rat1 [79] (Figure 2b). Budding yeasts depleted of Rio1, and human cells depleted of RIOK1 and the human orthologue of Rio1 exhibit an increase in CENP-A^{Cse4} levels, accumulation of centromeric transcripts and faulty recruitment of kinetochore components, which lead to chromosomal instability. If and how CENP-A increase directly promotes incorrect segregation was not determined.

Lamin A-associated proteins and the LINC complex

Two recent reports suggest emerging roles of Lamin A-associated proteins (LAAPs) and components of the LINC complex in centromere regulation. It was seen that in fission yeast Mis18 directly binds to Sad1, the major spindle pole body (SPB) linker of the nucleoskeleton and cytoskeleton (LINC) component, and that Sad1 is important for CENP-A^{Cnp1} deposition [80] (Figure 2c). This discovery was of particular interest as fission yeasts lack M18BP1 and their centromeres cluster at SPBs during G2, where and when CENP-A^{Cnp1} is reloaded. Similarly, a recent preprint describes the colocalization of Lamin A, BANF1 and Emerin – known to participate in the early events of nuclear envelope reformation along with other LAAPs – with the centromeres during telophase in human cells [81]. Depletion of each of these factors led to reduced recruitment of Mis18 α , M18BP1 and HJURP and to a drop in CENP-A, CENP-C and CENP-T levels with consequent increase in mitotic errors. The precise dependency between the mechanisms mediated by CENP-C and those putatively mediated by the LAAPs for Mis18C loading in telophase remains undetermined. Altogether, these data suggest an important role of the spatial organization of both centromeres and SPBs or LAAPs during centromere assembly. As it has been described that neocentromeres associate with distal heterochromatic regions and that centromeres cluster during interphase [82], it is possible that, in a similar fashion to transcription hubs, centromeric hubs could be assembled through the association to LAAPs to concentrate CENP-A deposition in few domains. This spatial separation could also be envisioned as an

additional mechanism of inhibiting the noncentromeric deposition of CENP-A. Both hypotheses should be formally addressed, for example by assessing the centromeric consequences of perturbing these 3D interactions.

Closing remarks

The main, canonical regulators of centromere assembly and maintenance have been well-known for many years. However, some of the molecular mechanisms regulating this complex process are still missing or have only recently been addressed. Ongoing efforts in the field aimed at unveiling all the details of this vital cellular process, combined with the realization that centromeres are more than just a platform for kinetochore assembly, led to the identification of new, unexpected, and often controversial, factors that contribute to the epigenetic inheritance of centromeres. It is likely that factors other than those mentioned here, such as chromatin remodelers, histone modifiers or DNA methylation, may also emerge as important players in centromere specification. Further investigation is required to ascertain the implication of each of these new factors and to incorporate them mechanistically into the established pathways of assembly and maintenance of the active centromeres.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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