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The ins and outs of CENP-A: Chromatin dynamics of the centromere-specific histone

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ABSTRACT

Centromeres are highly specialised chromosome domains defined by the presence of an epigenetic mark, the specific histone H3 variant called CENP-A (centromere protein A). They constitute the genomic regions on which kinetochores form and when defective cause segregation defects that can lead to aneuploidy and cancer. Here, we discuss how CENP-A is established and maintained to propagate centromere identity while subjected to dynamic chromatin remodelling during essential cellular processes like DNA repair, replication, and transcription. We highlight parallels and identify conserved mechanisms between different model organism with a particular focus on 1) the establishment of CENP-A at centromeres, 2) CENP-A maintenance during transcription and replication, and 3) the mechanisms that help preventing CENP-A localization at non-centromeric sites. We then give examples of how timely loading of new CENP-A to the centromere, maintenance of old CENP-A during S-phase and transcription, and removal of CENP-A at non-centromeric sites are coordinated and controlled by an intricate network of factors whose identity is slowly being unravelled.

1. Introduction

Centromeres act as a platform for the assembly of the kinetochore to ensure proper chromosomal segregation during cell division. In most eukaryotic cells, centromeres are specified epigenetically by the presence of a specific histone H3 variant called CENP-A (centromere protein A). Despite the importance of an epigenetic mark, the contribution of a genetic component cannot be excluded, as centromeric DNA or genomic regions associated with neocentromere formation have been shown to facilitate centromere formation and ultimately promote CENP-A loading [1–7]. Centromeres are highly specialised chromosome domains and when defective cause segregation abnormalities that can lead to aneuploidy and cancer. Timely loading of new CENP-A to the centromere, maintenance of old CENP-A during S-phase, and removal of CENP-A at non-centromeric sites is coordinated and controlled by an intricate network of factors whose identity is slowly being unravelled. Recent advances in chromatin biology are highlighting how chromatin remodelling is central to most processes occurring within the nucleus like DNA repair, replication, and transcription. How their role intersects with establishing and maintaining centromere identity is now becoming apparent. In this review, we will summarise recent advances on understanding the propagation of the centromeric histone variant CENP-A in the context of a dynamic chromatin environment. While highlighting parallels and identifying conserved mechanisms, we focused most of this work on a limited set of organisms, namely humans, the fruit fly, fission and budding yeasts. The first part of this review is organised in three broad sections that cover the establishment of CENP-A at centromeres, CENP-A maintenance during transcription and replication, and the mechanisms that help preventing CENP-A localization at non-centromeric sites. We then give examples of how chromatin remodelling participates in centromere biology and conclude by discussing how all these processes come together to control CENP-A dynamics.

2. Establishment (Loading)

CENP-A was initially discovered in patients with CREST syndrome [8] and was later found to be a histone protein related to histone H3 [9]. Subsequent works showed that CENP-A is sufficient to specify a centromere independently of the underlying DNA sequence [10–12]. CENP-A shares less than 60% sequence conservation with H3 in humans and is highly divergent between organisms, with most of the sequence variability lying in its N-terminal region. Despite these differences, CENP-As from distant organisms show a high degree of functional

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conservation, as exemplified by the ability of *S. cerevisiae* Cse4 to complement a knock-down of human CENP-A [13]. A particular region within the histone fold domain of CENP-A, namely CENP-A Targeting Domain (CATD), is required and sufficient for centromere targeting [14–16]. In humans, the CATD is composed of the loop L1 and alpha-helix α2 of CENP-A, and forms the recognition surface for many key factors involved in centromere biology, such as the specific CENP-A chaperone HJURP, and CENP-C and CENP-N [17–20], members of the Constitutive Centromeric Associated Network (CCAN). In addition, CENP-C recognises the extreme 6 amino acids at the C-terminal of CENP-A [18]. The CCAN is composed of 16 proteins in humans that are associated with the centromere throughout the cell cycle. It functions as a platform for the assembly of the remaining components of the kinetochore, the KNL-1/Mis12 complex/Ndc80 complex (KMN) network, which attaches to the mitotic spindle.

In S-phase, inheritance of the parental histones to either one of the two newly synthetised DNA strands leads to replicative dilution by half and is counterbalanced by loading of new histones. In contrast to canonical histones, CENP-A is not loaded at this stage, and the relative gaps are filled by placeholder nucleosomes containing H3.3 (or the closely related sole H3-variant hht3 in fission yeast) [5,21]. Loading of new CENP-A occurs in the following phases of the cell cycle and is essential to maintain centromere identity. CENP-A loading outside of S-phase is a conserved feature in many organisms, however, the timing of this process appears to be species-specific, with CENP-A being loaded between late telophase and G1 in humans, metaphase to G1 in Drosophila, and G2 in Arabidopsis and fission yeast [5,21–28]. CENP-A specific chaperones like HJURP in humans, its homolog Scm3 in fungi, and the unrelated CAL1 in *Drosophila*, are essential for CENP-A targeting to the centromere [29–33]. The available crystal structures of HJURP, Scm3 (from *K. lactis*), and CAL1 show that these chaperones form extensive contacts with the CATD of CENP-A when in the soluble CENP-A-H4 heterodimeric and pre-nucleosomal form [19,34,35]. Because localization of either HJURP, Scm3 or CAL1 to chromatin is

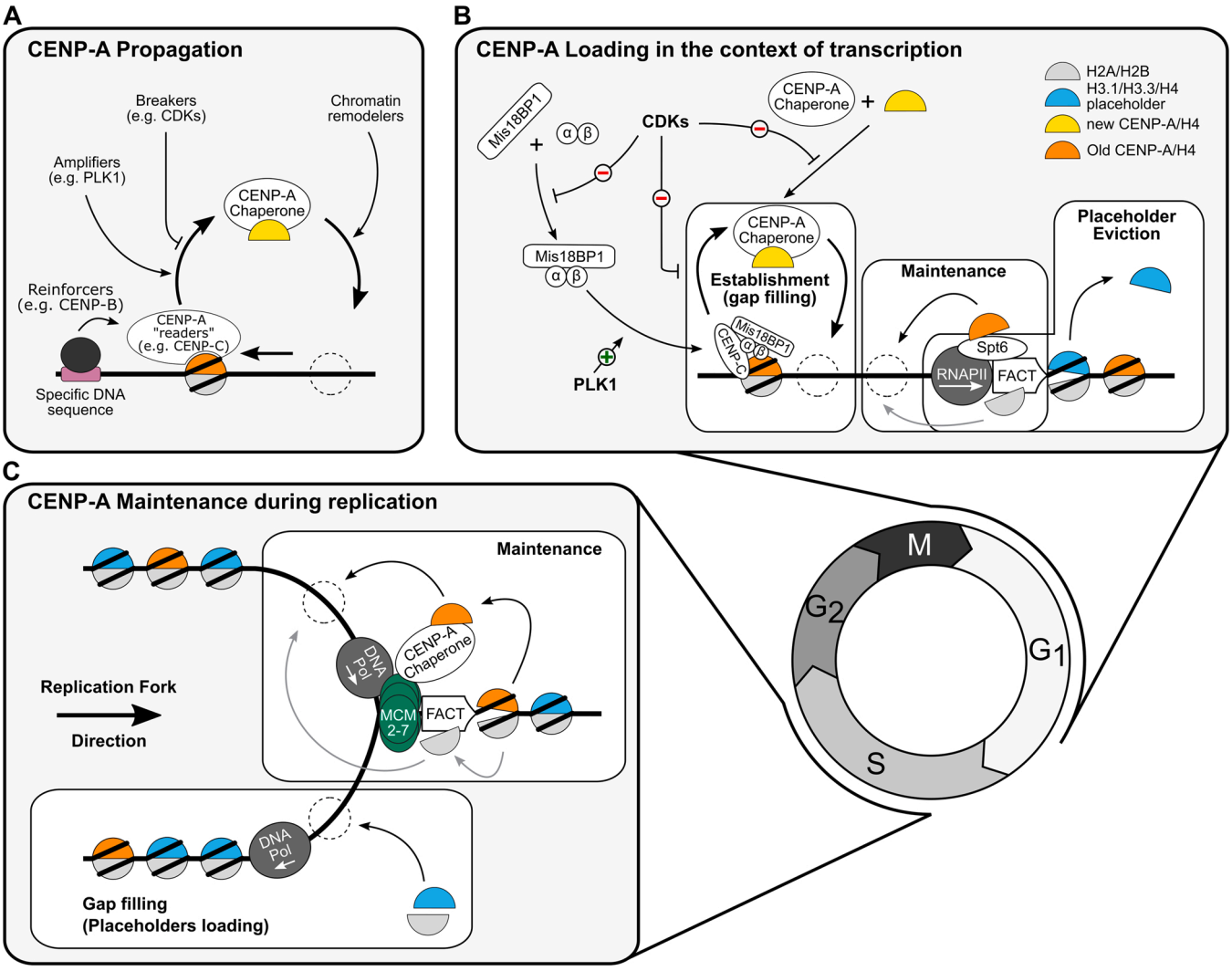

Fig. 1. Mechanisms controlling CENP-A establishment and maintenance. **A)** Epigenetic feedback loop for CENP-A propagation at the centromere. Specific CENP-A "readers" (i.e. CENP-C) recognise nucleosomal CENP-A and recruit specific CENP-A chaperones (e.g. HJURP, Scm3, CAL1). This chaperone leads to loading of CENP-A, effectively acting as a "recruiter/writer" that deposits the centromeric information into chromatin. In addition, genetic mechanisms can reinforce the loop by stabilising the centromeric CENP-A "readers". Finally, other signals can amplify or break this loop. **B)** Model describing CENP-A loading in the context of transcription-induced chromatin remodelling in vertebrates. Centromeric transcription by RNA polymerase II promotes placeholder nucleosomes eviction, while already loaded CENP-A nucleosomes are maintained by Spt6 and FACT through reassembly in the wake of the polymerase. The gaps generated by this remodelling are filled with new CENP-A. The assembly and centromeric localization of the Mis18 complex and the CENP-A chaperone is restricted to a specific time window by regulatory kinases CDKs and PLK1. **C)** CENP-A maintenance during replication. The interaction between HJURP and the MCM2–7 helicase complex ensures that CENP-A is reassembled during replication and inherited by the newly synthetised strands. CENP-A replicative dilution is counteracted by the loading of placeholder nucleosomes.

sufficient to initiate CENP-A loading and centromere formation, additional cell cycle regulated licensing mechanisms need to be in place to prevent spurious recruitment of CENP-A to chromatin.

In vertebrates, targeting of HJURP-CENP-A-H4 to the centromere occurs through the interaction with the Mis18 complex, composed of Mis18α, Mis18β, and Mis18BP1. Mis18α and Mis18β transiently interact with Mis18BP1 to form the Mis18 complex, which can interact with both CENP-C [36–38] and the centromeric CENP-A nucleosome [36,39] on one side, and with HJURP-CENP-A-H4 on the other (Fig. 1B) [40]. Interactions between these various components are tightly regulated during the cell cycle to ensure that CENP-A loading occurs once per cycle. The cyclin-dependent kinases 1 and 2 (CDK1/2) phosphorylate HJURP and Mis18BP1 during S/G2, preventing centromere localization and limiting CENP-A deposition to late telophase/G1, when CDKs activity is at their minimum [41–43]. Phosphorylation by CDK1 and 2 of Mis18BP1 also disrupts the interaction with both Mis18α/Mis18β and the centromere, further inhibiting CENP-A deposition [42]. Polo-like kinase 1 (PLK1) promotes CENP-A loading and Mis18BP1 localization at the centromere acting as an antagonist to the CDKs by phosphorylating Mis18BP1 at other residues [44]. In a recent study, the human SUMO-peptidase SENP6 has also been identified as a regulator of CENP-A localization by protecting Mis18BP1. SENP6 counteracts the activity of the SUMO ligase PIAS4, which was shown to sumoylate Mis18BP1 and promote its degradation through the SUMOylation-dependent ubiquitin ligase RNF4 [45]. Similarly, the deubiquitinase USP11 protects HJURP and facilitates its interaction with CENP-A [46]. The chaperone proteins RbAp46 and RbAp48, which are part of many chromatin associated complexes (e.g. NURF, NURD, PRC2), have also been proposed to prime CENP-A loading by acting in conjunction with HAT1 to acetylate H4 at residues Lys5 and Lys12 in the CENP-A-H4 prenucleosomal complex [47]. In addition to HJURP and the Mis18 complex, several post-translational modifications on CENP-A have been proposed to regulate centromere targeting and loading. Phosphorylation of Ser68 has been suggested to weaken the binding to HJURP, and a recent report linked it also to CENP-A degradation (see Section 4) [48–51]. Phosphorylation of Ser18, initially identified in a high-resolution mass spec experiment [52], was recently found to affect CENP-A stability at the centromere [53]. By tethering mCherry-LacI-HJURP to LacO repeats, Takada and colleagues observed that a CENP-A S18D mutant could still be recruited by HJURP but a large portion of the protein was mislocalised. The authors therefore speculated that S18D might weaken the binding to HJURP. In addition to the epigenetic loop described earlier, a genetic mechanism is known to contribute to CENP-A loading. The protein CENP-B binds to short DNA motifs in the alpha satellite repeats and stabilises CENP-C at centromeres [54,55]. Despite not being essential for CENP-A loading and centromere formation [56–58], cells lacking CENP-B have reduced CENP-A [59], indicating a parallel DNA-dependent mechanism that promotes the epigenetic loop formed between CENP-A, CENP-C, HJURP, and the Mis18 complex.

In *Drosophila*, HJURP and the Mis18 complex are not conserved and instead the protein CAL1 was proposed to combine both the CENP-A chaperone and centromere targeting activities in one protein. It was shown to recruit dCENP-A (also known as centromere identifier, CID), establish functional centromeres and, together with dCENP-C, sufficient to propagate dCENP-A in a heterologous system [33,60,61]. Despite the fact that HJURP and CAL1 appear to be unrelated [35,60], recent crystal structures have shown that the N-terminus of CAL1 binds dCENP-A through specialised contacts similar to HJURP. Interestingly, CAL1's C-terminus engages directly with the conserved cupin dimerization domain of dCENP-C [33,35,61,62], suggesting that CAL1 combines both activities of HJURP and Mis18. Several additional factors are known to regulate dCENP-A loading. The FACT complex interacts with CAL1 and is required for loading [63]. The histone chaperone p55(RbAp48) is also proposed to be implicated in dCENP-A deposition by chaperoning dCENP-A-H4 and assembling it on chromatin [64,65].

In the holocentric nematode *Caenorhabditis elegans*, multiple centromeres are assembled across the whole length of the chromosome [66,67]. Several of the factors that load CENP-A are not conserved, such as the Mis18α/β subunits of the Mis18 complex, thus licensing depends on KNL-2, the orthologue of MIS18BP1 [68]. Although a specific CENP-A chaperone appears to be absent in *C. elegans*, it was recently reported that the extreme N-terminal region of worm CENP-A partially resembles HJURP and might fulfil the chaperone's functions by mediating the interaction with KNL-2 [69,70]. The histone chaperone LIN-53 (RbAp46/48 in humans) is also required for CENP-A loading. In contrast to humans, HAT1 acetylase activity is not necessary [71], but *de novo* centromere formation on artificial chromosomes requires both HAT1 and the condensing I/II subunit SMC-4 [72].

In *S. pombe*, the core components controlling $Cnp1^{CENP-A}$ deposition are mostly conserved. The Mis18 complex is composed of the subunits Mis18 (human Mis18α/Mis18β), Mis16 (RbAp46 and RbAp48 in humans), and Eic1 (a functional homolog of Mis18BP1) [47,73]. The activity of the $Cnp1^{CENP-A}$ chaperone Scm3, together with the Mis18 complex is required to target $Cnp1^{CENP-A}$ to the centromere [31,32,47]. Loading has been observed in G2 phase in fission yeast, and the CCAN/Mis6/Ctf19 complex, which forms part of the yeast kinetochore and is a known interactor of the Mis18 complex, has been proposed as a factor affecting the timing of $Cnp1^{CENP-A}$ loading [5,73–75].

In contrast to the examples above that rely on epigenetic mechanisms, the point centromeres of *Saccharomyces cerevisiae* are defined genetically by the presence of a specific DNA sequence of about 125 bp, which contains three centromeric DNA elements (CDEI-III). Nonetheless they also contain the CENP-A homolog $Cse4^{CENP-A}$, which is assembled on this region in form of a single nucleosome. This is mediated through the activity of its chaperone Scm3, the protein Cbf1 that recognises the CDEI sequence, and the multi-subunit complex CBF3, which binds to CDEIII and is required for kinetochore assembly [29,76]. Unlike organisms with regional centromeres, localization of Scm3 depends on the interaction with the CBF3 complex and loading of $Cse4^{CENP-A}$ occurs in S-phase [76,77].

In summary, CENP-A targeting to the centromere appears to employ conserved strategies and similar factors in distant organisms. The core factors seem to be: 1) a specific CENP-A chaperone (e.g. HJURP, Scm3, CAL1, etc); 2) a protein or protein complex that localises to the centromere and can recruit the chaperone (i.e. CENP-C and Mis18 complex); 3) signals that act either to prevent or stimulate the recruitment and interaction between the previous two factors, as in the case of the inhibitory HJURP and MIS18BP1 phosphorylation by CDK1, or the promoting MIS18BP1 phosphorylation by PLK1. Other factors (e.g. CENP-B, etc) where present enable additional layers of regulation. While the chaperones (HJURP, Scm3, CAL1) are functionally conserved, the time-windows of the cell cycle in which CENP-A is assembled have diverged. In organisms containing regional centromeres (e.g. humans, Drosophila, S. pombe, etc), loading of CENP-A outside the S-phase seems to be a general paradigm. This might be due to the promiscuity of many histone chaperones and the elevated amounts of H3 during replication. Restricting loading outside S-phase might help preventing unspecific chaperone-histone interactions due to competition between H3/H3.3 and CENP-A [78] and provides a more specific mean to control CENP-A incorporation. This would help preserving centromere identity and avoid dilution of the centromeric mark or creating ectopic kinetochores. This cell cycle-controlled loading might be less important in budding yeast, where the centromere is encoded by a defined DNA sequence providing an additional level of control for CENP-A deposition.

Interestingly, as we gain a deeper understanding into how centromere identity is propagated alongside other cellular processes, centromeric transcription has emerged to play an important role in CENP-A loading. Transcription inhibition results in decreased CENP-A at centromeres [2,79–83], and transcription by RNA polymerase II and concomitant chromatin remodelling are required to mediate the transition of CENP-A from a chromatin-associated state to a stably

incorporated form [84]. As transcription-mediated chromatin remodelling can evict nucleosomes [85–88], it is proposed that centromeric transcription by RNA polymerase II leads to eviction of placeholder H3.3 nucleosomes, or H3 in fission yeast, and this makes room for incorporation of new CENP-A into chromatin [63,81,84,89–92]. However, unchecked transcription can destabilise incorporated CENP-A and lead to centromeric instability [93,94]. Centromeric RNA transcripts have also been proposed to have a role at the centromere by making direct contacts with CENP-A, HJUPR, CENP-C, and the kinetochore components [95–104], and depending on the organism, these transcripts have been observed to act either in *cis* or in *trans*. The fact that centromeres are transcribed in very distant organisms underlines the critical role it plays at centromeres, but further studies are required to unambiguously discern between the importance of remodelling versus nascent centromeric transcripts in CENP-A dynamics.

3. Maintenance (Recycling)

Nucleosomes throughout the genome are actively remodelled during both replication and transcription, including at the centromere. Consequently, it would be expected that these processes increase CENP-A mobility and challenge its epigenetic maintenance. However, CENP-A turnover at the centromere is extremely slow, as confirmed by the fact that in mouse oocytes CENP-A nucleosomes persist for over a year in prophase arrested cells [105]. Although, in quiescent cells, CENP-A is dynamic and needs to be constantly replenished [106], therefore, mechanisms to efficiently maintain already deposited CENP-A must be in place.

3.1. Transcription-coupled maintenance of CENP-A

Centromere transcription by RNA polymerase II is remarkable because it takes place in mitosis when the rest of the genome is transcriptionally silent. Although centromeric transcription is not required for progression through mitosis [107], it appears to play an important role for maintaining centromere identity [90,106]. Inhibition of transcription results in defective incorporation of CENP-A into nucleosomes [84]. As mentioned in Section 2, both the transcribed RNAs and transcription-mediated chromatin remodelling have been implied in CENP-A loading. Nucleosomes must be disassembled in front of the RNA polymerase to allow transcription, and they are reassembled in the wake of the polymerase to prevent loss of epigenetic information. The mechanism through which previously deposited CENP-A nucleosomes are retained while placeholders are removed is still unclear. Two main histone chaperones have been linked to nucleosome maintenance during transcription: the Facilitates Chromatin Transcription (FACT) complex, and the transcription elongation factor Spt6 [108,109]. Both chaperones are involved in general histone recycling across the genome. Spt6 is a histone chaperone that travels together with RNA Pol II, and its N-terminal region is capable of directly binding both H3/H4 and dCENP-A/H4 in vitro [90,110–112]. In co-immunoprecipitation experiments from *Drosophila* S2 cells extracts, Spt6 was shown to preferentially bind dCENP-A over histone H3, with the interaction depending on both the nucleosome core and tail of dCENP-A. Phosphorylation of the N-terminal tail (S77) of dCENP-A appears to negatively affect the interaction with Spt6 and ultimately results in defective maintenance of dCENP-A at the centromere [65,90]. While the particular phosphorylation site is not conserved in mammals, phosphorylation of the N-terminal tail of CENP-A (S30) in murine cells has also been linked to centromeric removal of CENP-A under stress [113] and a similarly positioned phosphorylation (S31) has been identified on human CENP-A [114] This together with the fact that SPT6 depletion in HeLa cells results in defective CENP-A maintenance raises the interesting possibility that a phospho-dependent switch might be regulating SPT6/CENP-A interaction [90].

3.2. Maintenance during replication

Similar to DNA transcription, chromatin needs to be partially disassembled during S-phase to allow the DNA to be replicated by the DNA polymerases. Parental histones are inherited by either one of the newly synthetised strands, while the resulting gaps on the other are filled by de novo nucleosome assembly. Interestingly, HJURP was recently shown to interact with the MCM2 subunit of the DNA helicase MCM2–7 complex and facilitate recycling of parental CENP-A at the replication fork (Fig. 1C) [115]. MCM2-HJURP interaction is reminiscent of MCM2-Asf1α, which is involved in maintaining canonical H3 [116,117]. MCM2 and HJURP are suggested to bind CENP-A simultaneously, with MCM2 recognising the R63-K64 motif conserved in all H3-variants, while HJURP binds the CENP-A centromere targeting domain (CATD) [115,118]. However, both HJURP and MCM2 are predicted to compete for binding to the helix α1 of CENP-A, raising the possibility of a different mode of interaction. MCM2 can bind both histone H3 variants-H4 heterotetramers and heterodimers, while HJURP binds CENP-A-H4 as a dimer [19,116–118]. Therefore, a sequential mechanism has been proposed where MCM2 captures a CENP-A-H4 tetramer which is then split and bound by HJURP as dimers. The capacity of HJURP to dimerise might provide a mean to bring both CENP-A-H4 dimers back together and recycle the whole tetramer [117,119]. While this model is intriguing, there is evidence that additional factors are required for CENP-A maintenance at the centromere during replication. Several studies have found that the majority of CENP-A-containing nucleosomes (60%) is found outside the centromere [120,121]. Since the presence of CENP-A at non-centromeric sites can lead to the formation of ectopic centromeres, several mechanisms act in concert to prevent CENP-A accumulation at these sites (see Section 4). Amongst these processes, the DNA replication machinery has been shown to remove ectopically incorporated CENP-A from chromosome arms without affecting that deposited at the centromere [121]. Since removal of CENP-C during S-phase leads to massive loss of CENP-A at the centromere [121], Nechemia-Arbely et al. hypothesise that the CCAN directly participates in CENP-A maintenance. How CENP-A is maintained during replication in other organism and to learn whether the MCM complex interaction with CENP-A specific chaperones is a conserved mechanism will require additional studies.

4. Sculpting CENP-A distribution

Genome wide analysis have consistently found a large proportion of CENP-A at ectopic sites in the genome, indicating that the mechanisms controlling CENP-A loading are not sufficient to restrict CENP-A to centromeres [120]. Although there a fewer CENP-A nucleosomes than H3 nucleosomes at the centromere (1:25 respectively), CENP-A is still enriched ~50 folds more at the centromere than the rest of the genome. Based on this observation, it has been proposed that a critical local concentration of CENP-A is required for the assembly of kinetochores [120]. In accordance, CENP-A localised to the chromosome arms under physiological expression levels does not lead to the assembly of functional ectopic kinetochores. However, overexpression of CENP-A dramatically promotes its mislocalization [122,123] and the capacity to assemble ectopic kinetochores [124–133]. This indicates that the amounts of CENP-A available for nucleosome incorporation must be tightly regulated in the cell to ensure the centromeres are properly assembled. Indeed, several mechanisms have been found to contribute to correct CENP-A loading at the centromere and prevent accumulation at ectopic sites. Among these processes, both transcriptional and post-transcriptional mechanisms are known to control CENP-A dynamics. By acting together, they are capable to prevent deposition of CENP-A at ectopic loci, while actively promoting removal of mislocalised CENP-A, thereby ultimately “sculpting” chromatin to achieve enrichment of CENP-A exclusively at the centromere.

4.1. Transcriptional regulation

Transcription of the CENP-A gene is closely associated to the timing of its loading in some organisms and appears to be uncoupled from canonical histones transcription. In humans, CENP-A transcription occurs during G2/M phase [134], while in fission yeast it occurs in G1 phase [135,136], prior to the respective window in which CENP-A is loaded. Several factors directly controlling transcription of the CENP-A gene have been recently identified in various organisms. In human cells the protein Cdk5rap2 interacts with the promoter region of CENP-A and upregulates its transcription (Fig. 2A, top panel) [137]. In fission yeast, the *MluI box-binding factors* (MBF) complex binds to the *MluI cell cycle box* (MCB) and acts as a transcriptional repressor restricting $Cnp1^{CENP-A}$ transcription to G1 (Fig. 2A, bottom panel) [136]. Although, using different promoters to drive $Cnp1^{CENP-A}$ transcription, Aristizabal-Corrales et al. showed that cells expressing $Cnp1^{CENP-A}$ outside G1 could still assemble it at centromeres, $Cnp1^{CENP-A}$ levels directly correlated with the amount of ectopic incorporation [136]. Similar observations have been made in other organisms [124–128, 131–133], indicating that while cells with reduced or slightly higher levels of CENP-A can still be functional, there appears to be a threshold over which the cells are no longer capable of compensating. This results in local enrichment of CENP-A at non-centromeric sites, promotes ectopic kinetochore formation and, consequently, mitotic defects.

4.2. 4.2 Post-translational regulation

Several post-translational mechanisms are known to control or "sculpt" CENP-A localization. In *Drosophila*, dCENP-A protein levels are maintained low during G1/S phase by the E3 ubiquitin ligases SCF^{Ppa} and APC/C^{Cdh1} [133]. Acting in the opposite direction, another ubiquitin ligase, namely CUL3/RDX, monoubiquitinates CENP-A when in complex with CAL1 and leads to CENP-A stabilization [138]. Interestingly, the specific interaction of dCENP-A with the chaperone CAL1 also prevents SCF^{Ppa} -mediated degradation [133]. Protection against dCENP-A degradation through CAL1 binding has been proposed to limit the availability of free dCENP-A for other non-specific chaperones, promoting dCENP-A loading specifically at the centromere [138]. Moreover, phosphorylation of dCENP-A Ser20 by casein kinase II (CKII) has been linked to its centromeric enrichment, turnover at ectopic sites, and pre-nucleosomal stability. S20ph acts upstream of SCF^{Ppa}, and it is required for dCENP-A degradation in its pre-nucleosomal form. S20ph seems also to promote dCENP-A loading on chromatin. However, while phosphorylated dCENP-A is stably incorporated at centromeres, S20ph leads to increased dCENP-A turnover and removal at ectopic sites [139]. These seemingly contradicting behaviours might indicate a mechanism that protects dCENP-A from degradation specifically at the centromere, possibly through the association with centromere-specific factors like dCENP-C.

In humans, despite the limited evidence, similar mechanisms that stabilise or destabilise CENP-A appear to be controlling CENP-A dynamics. Preliminary studies suggest that CENP-A degradation is controlled by ubiquitination in senescent cells [140]. Moreover, herpes simplex virus type 1 protein ICP0 is known to promote CENP-A proteolysis in infected cells [141]. As discussed further above, PTMs of CENP-A also play an important role to regulate its function, including

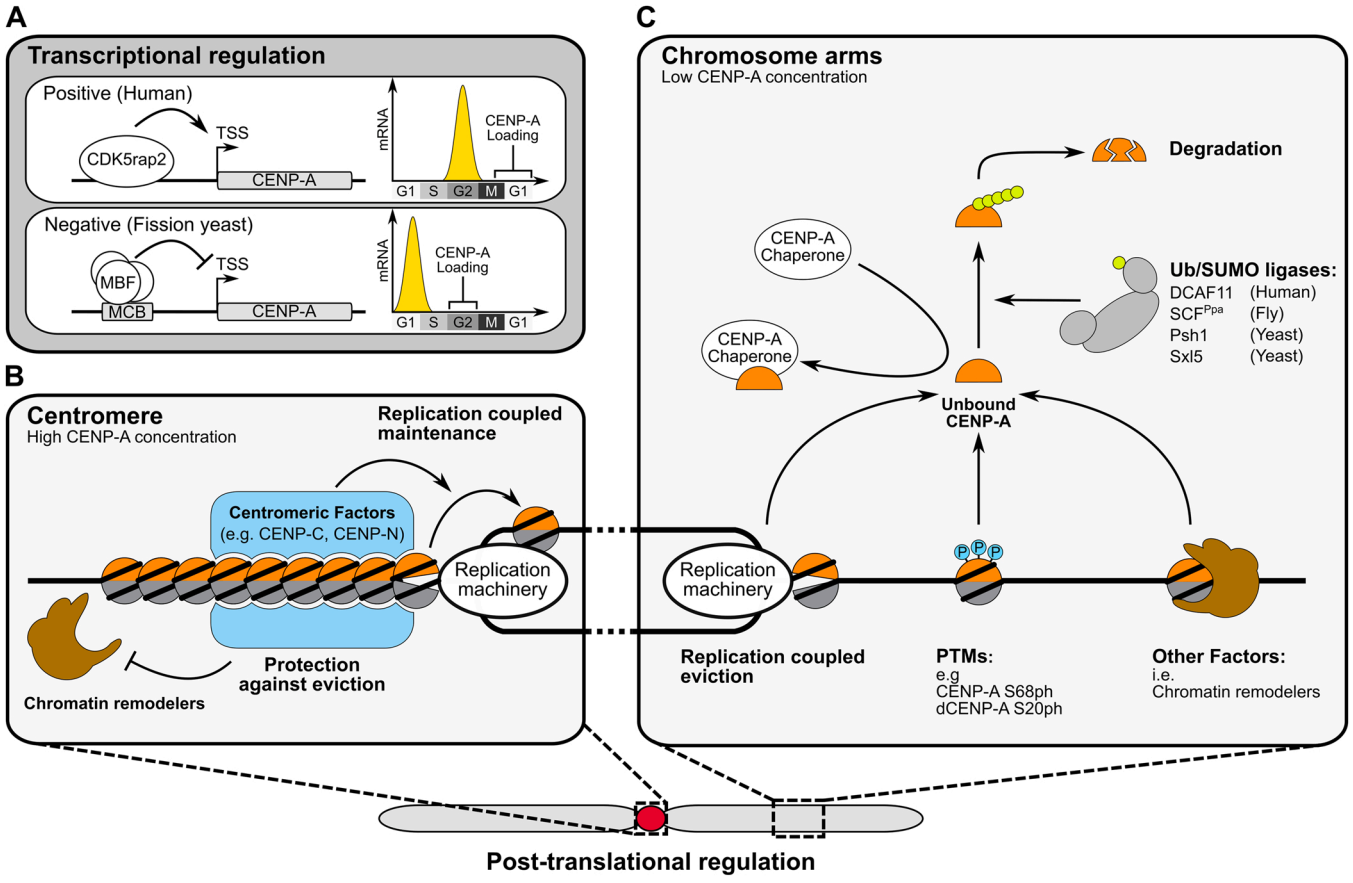

Fig. 2. Mechanisms sculpting CENP-A localization. **A)** Transcriptional regulation of the CENP-A gene through either positive or negative signals achieves CENP-A expression to a time window closely associated to CENP-A loading. **B-C)** Post-translational mechanisms ensure that CENP-A is enriched at the centromere. Ectopic CENP-A is targeted by multiple processes that mark it for removal through both replication dependent and independent mechanisms. At the centromere, specific centromeric factors (e.g. CENP-C, CENP-N) protect CENP-A from eviction, resulting in higher local concentration of CENP-A.

protein stability. The role of CENP-A Ser68 phosphorylation/dephosphorylation by Cdk1/PP1α has been debated for some time [48–51]. It has been recently reported that the presence of S68ph promotes polyubiquitination of CENP-A at Lys49 and Lys124 by the E3 Ubiquitin ligase DCAF11 during mitosis [51]. In the proposed model, Cyclin B-Cdk1 phosphorylates S68 in G2/early-M phase immediately after CENP-A is translated, and this leads to its degradation during mitosis, preventing ectopic mislocalization similar to SCF^{Ppa} in *Drosophila*. Interestingly, S68A and K49R/K124R double mutant do not show evident phenotypes in cycling cells. Nevertheless, when arrested in early S-phase by aphidicolin or thymidine treatment, these mutant cells show accumulation of CENP-A at ectopic sites, in line with the recent report that the DNA replication machinery protects centromere identity by removing mislocalised non-centromeric CENP-A [51,121]. Nechemia-Arbely et al. followed the deposition of CENP-A during the cell cycle and found that CENP-A was loaded across the genome during G1, but all the non-centromeric CENP-A had been removed in the next G2 phase. They further show that most of the ectopic CENP-A sites are replicated in early-S, while centromeres are replicated in late S-phase. By tracking replication and CENP-A localization they also observe that non-centromeric CENP-A is evicted by the replication fork, while centromeric CENP-A is maintained in the same spots. Finally, the authors show that the CCAN, and in particular CENP-C, remain associated during replication and are essential to protect CENP-A from the replication machinery at centromeres [121]. These results confirm the previously described protective role that CENP-C and CENP-N have on CENP-A nucleosomes [142,143].

Also in budding yeast, proteolysis is important to regulate $Cse4^{CENP\text{-}A}$ levels and prevent ectopic localization. Ubiquitination of the N-terminal tail of $Cse4^{CENP\text{-}A}$ by Doa1, and ubiquitination of $Cse4^{CENP\text{-}A}$ CATD by the E3 ubiquitin ligase Psh1 lead to $Cse4^{CENP\text{-}A}$ degradation [144–147]. The activity of Psh1 is antagonised by Ubp8, a deubiquitinase enzyme part of the SAGA complex, whose activity prevents excessive Cse4 proteolysis, mitotic instability, and Cse4 mislocalization [148]. In a mechanism reminiscent of the interdependent CAL1-CENP-A stability, Cse4 is also protected from degradation when in complex with both Scm3 and the kinetochore protein Pat1 at the centromere [147]. Interestingly, when Psh1 is deleted, Cse4 accumulates at intergenic regions in proximity of nucleosome depleted regions such as the + 1 nucleosome at promoters, replication origins, and centromeres. This mislocalization to euchromatic regions requires the remodelling activity of the Ino80 complex and alters transcription at the affected loci [149]. Several other ubiquitin ligase components are known and appear to participate in preventing Cse4 mislocalization [150–152]. In addition, The SUMO targeted ubiquitin ligase (STUbL) protein Sxl5 sumoylates K65 on Cse4, modification that is critical for Cse4 proteolysis and to prevent mislocalization [153].

Other factors known to prevent CENP-A from deposition at ectopic sites include the histone variant H2A.Z [154], the chromatin remodellers CHRAC and SWI/SNF [155,156], and the chaperones FACT and Spt6 [157,158]. For a more extensive analysis of CENP-A dynamics at non-centromeric site we suggest the recent review by Dong et al. [159].

In summary, CENP-A protein levels are tightly regulated in most organisms. Ubiquitin-mediated degradation seems to be a common mechanism employed to prevent overexpression and mislocalization of CENP-A, and eventual non-centromeric CENP-A appears to be removed in a replication-coupled mechanism (Fig. 2B). This requires also antagonistic mechanisms to be in place to prevent CENP-A from being completely degraded and removed. The interactions between the specific CENP-A chaperones and CENP-A seem to be the most conserved mechanism to ensure centromeric loading and protect CENP-A from proteolysis. In addition, the centromere location and more specifically members of the CCAN appear to protect CENP-A at the centromere from eviction and degradation. It would be interesting to investigate if this is conserved in organisms other than humans and yeast, specifically those where many CCAN members are absent. Overall, the current knowledge supports a model in which CENP-A localization depends on a combination of mechanisms specifically targeting CENP-A at the centromere, and processes required to sculpt and restrict CENP-A at centromeres by removal of non-centromeric CENP-A.

4.3. CENP-A localization at DNA damage sites

Among the non-centromeric sites where CENP-A becomes recruited, DNA double-strand breaks (DSBs) represent a particularly interesting case. CENP-A has been repeatedly shown to be deposited at DSBs together with CENP-N, CENP-T, and CENP-U, and its recruitment seems to correlate with the activity of the non-homologous-end-joining pathway (NHEJ) [160–162]. Since this recruitment is independent of Ligase IV, DNA-PKcs, and H2A.X, it is likely that CENP-A localises to DSBs at early stages of repair [162]. In addition to a potential link to the NHEJ pathway, a recent report showed that CENP-A is capable of recruiting key proteins of the homologous recombination (HR) repair pathway to induced DSBs [46], suggesting a connection between CENP-A and the DNA damage repair (DDR) pathways. Intriguingly, transcription and DNA repair are also linked, as transcription appears to recruit DDR proteins [163]. It is tempting to speculate that part of the ectopic CENP-A might derive from an increased presence of DSBs at these regions in a transcription dependent manner. Targeting of CENP-A to these sites might be mediated by the chaperone complex ATRX-DAXX, which has been found to bind CENP-A and to work in both the NHEJ and HR pathway [78,164,165]. Further studies are required to understand if there is a causal link between transcription-DNA repair and the CENP-A found at ectopic sites.

5. Chromatin remodelling at the centromere

As discussed above, processes that involve direct access to DNA, require chromatin to be remodelled. The presence of nucleosomes imposes a physical barrier particularly to the replication and transcriptional machineries. To circumvent these obstacles, a plethora of protein complexes are known to control chromatin dynamics through several mechanisms. By depositing posttranslational modifications on histones, nucleosome-nucleosome interactions can be directly disrupted or promoted. These PTMs can be also recognised by additional proteins that in turn can lead to chromatin opening or compaction, either directly or indirectly (e.g. HP1, PRC2, etc). In addition, ATP-dependent chromatin remodellers use energy to alter the interaction between histones-DNA within a nucleosome. Based on the domain composition of the core ATPases, these remodellers are classified in four subfamilies: CHD, SWI/SNF, ISWI, INO80. Yet, the activity of these complexes can be summarised in three main types: 1) nucleosome editing [CHD, INO80], in which the histone composition of a nucleosome is changed; 2) chromatin opening [CHD, SWI/SNF], in which spacing between nucleosomes is altered by either nucleosome sliding, eviction, or partially disassembling nucleosomes; 3) chromatin assembly [ISWI, CHD], in which nucleosomes are properly assembled and moved around to generate regularly spaced arrays [166]. All the aforementioned factors control the dynamic chromatin remodelling processes with histone chaperones and in concert with physiological processes like DNA replication, DNA repair and transcription.

Increasing evidence supports a pivotal role for chromatin remodelling at the centromere. As discussed in the establishment section (Section 2), loading of CENP-A outside S-phase results in centromeres interspersed with placeholder nucleosomes containing H3 or H3.3 [5,21]. These nucleosomes are later removed to allow loading of new CENP-A, however, the molecular details of this transition are not understood. Likewise, the above discussed hypothesis that transcription-mediated chromatin remodelling allows the removal of placeholder nucleosomes likely involves the activity of chromatin remodellers. While further investigation is required to support this model, there is already a growing number of studies that link chromatin

remodellers to CENP-A dynamics. In a recent RNAi screening, multiple factors have been found to affect the deposition and maintenance of CENP-A [167]. Knockdown of several components of the transcriptional machinery or the remodellers SMARCAD1, SMARCD3, and the histone chaperone ASF1B resulted in decreased deposition of new CENP-A. Similarly, depletion of the remodellers SMARCAD1, ACTL6B, CHD8, and HLTF led to defective maintenance of CENP-A. Interestingly, most of the remodellers identified belong to the SWI/SNF subfamily of chromatin remodellers (SMARCAD1, ACTL6B, HLTF, and SMARCD3), and SMARCAD1 plays a role in both CENP-A establishment and maintenance. SMARCAD1 in humans, and its orthologues in other organisms have also been associated to both the DNA damage response and transcription. The fission yeast homolog of SMARCAD1/Fft3 was shown to ensure epigenetic inheritance by controlling histone turnover at heterochromatic regions [168,169]. Fft3 has been reported to work in concert with FACT to disassemble nucleosomes in front of RNA pol II, *de facto* promoting elongation by reducing the nucleosome barrier [170]. Intriguingly, Fft3 is enriched at the centromere and at heterochromatic insulator sites, and it prevents euchromatinization of these regions, as indicated by the increased H2A.Z and H4K12ac levels in a fft3Δ mutant background [171]. Similarly, in budding yeast, the SMARCAD1 homologue Fun30 promotes correct chromatin structure around the point centromeres [172]. The remodellers contribution to centromere is not limited to the SWI/SNF subfamily. The RSF complex is an ISWI remodeller, composed of the ATPase SNF2h and RSF1, which has been implicated in centromeric chromatin maintenance and DNA repair. An initial link between CENP-A and RSF was identified in ChIP-mass spec experiment of enriched CENP-A chromatin [173]. A later report further investigated this link and found that RSC is enriched at centromeres during mid-G1, and that depletion of both RSF subunits leads to decreased CENP-A loading and prometaphase defects [174]. Moreover, recruitment of CENP-A to DNA double-strand break sites depends on the activity of RSF, however, evidence of a physical interaction during this process is lacking [160,161]. In fission yeast, the Ino80 complex is required for proper centromere establishment by promoting remodelling and removal of H3 during transcription [91,175,176]. Finally, two additional studies suggested a role for the CHD remodellers CHD1 and Hrp1 in vertebrates and fission yeast, however, this does not seem to be conserved in *Drosophila* [177–179].

6. Discussion

The histone H3-variant CENP-A is central to the formation of centromeres and functional kinetochores and perturbations of its dynamics can lead to disastrous outcomes. Not surprisingly, an increasing number of studies in recent years have linked CENP-A to developmental diseases and cancer. It is therefore essential to understand the mechanisms that establish, maintain, and restrict CENP-A to the centromere. Our current knowledge indicates that three main processes act together to control CENP-A localization: loading, maintaining, and sculpting. CENP-A is recruited and loaded at the centromere through an epigenetic mechanism that relies on the presence of previously deposited CENP-A. The factors involved in this step are CENP-C, HJURP (Scm3 in yeast, CAL1 in *Drosophila*), and the Mis18 complex (or relative orthologues), which together are capable to “read” the position of the centromeric CENP-A and recruit new CENP-A for loading. Additional factor, such as PLK1 and CDK1, act as positive or negative regulators respectively for licensing this process during specific cell cycle phases (Fig. 1). Still, this epigenetic loop is not sufficient to explain the fact that CENP-A can also be found in thousands of non-centromeric loci, as observed before replication in humans [120,121]. To restrict CENP-A to the centromere, a “sculpting” process appears to be in place that removes CENP-A, mostly from non-centromeric regions [176]. The replication machinery appears to be key in this process by evicting ectopic CENP-A. Initial evidence indicates that the CCAN protects centromeric CENP-A from being removed during replication, possibly suggesting that its absence on the chromosome arms might suffice to distinguish these regions from the centromere (Fig. 2B). It is possible that the absence of the protecting CCAN generates the opportunity to deposit PTMs on the non-centromeric CENP-A destined for removal. The presence of these PTMs could then be signals for removal by the replication machinery. In addition, these PTMs could also prevent CENP-A accumulation outside S-phase through transcription dependent or independent chromatin remodelling (Fig. 2C). How these PTMs are deposited and regulate CENP-A during the cell cycle will require to understand the molecular underpinnings of CENP-A removal.

The localization of CENP-A at ectopic sites when CENP-A is expressed at endogenous levels raises the additional question of whether this has a biological role and, if so, which are the factors that lead to this non-centromeric localization. Details about this process are lacking, however, several factors have been implied in CENP-A mislocalization when this is overexpressed. The histone chaperone complex ATRX-DAXX specifically binds H3.3, however, it can also bind CENP-A when present at elevated levels. This interaction leads to the assembly of heterotypic nucleosomes containing both H3.3 and CENP-A, which have been found enriched at regions of high histone turnover and transcription factor binding sites [78]. Interestingly, Nechemia-Arbely et al. found ectopic CENP-A at similar sites when not overexpressed [121]. Most identified sites overlapped with the characteristic H3K4me2 marker that is essential for loading of new CENP-A on a human artificial centromere [180]. Moreover, CENP-A was generally found at regions containing also marks of active transcription, such as H3K4me3, H3K36me3, and H2A.Z [121,180]. Transcription-induced chromatin remodelling is essential for loading CENP-A at the centromere, and it is therefore intriguing to speculate that a similar mechanism might drive CENP-A to non-centromeric sites. Recruitment of CENP-A to DSBs, which have been found enriched at transcriptionally active regions, might also play a role in CENP-A delocalization, however additional studies are required to support these hypotheses.

7. Concluding remarks and future perspectives

The growing body of studies about CENP-A dynamics at the centromere and beyond are revealing an intricate network of processes required to specify and maintain centromeres. Increasing evidence indicates the pivotal role played by chromatin remodelling in ensuring CENP-A loading at the centromere and removal on the chromosome arms. However, many questions remain unanswered. Among the most prevalent ones are the molecular processes that remove CENP-A from non-centromeric sites by the DNA replication machinery and other chromatin-remodelling processes, how transcription-mediated chromatin remodelling promotes loading of CENP-A but removal of placeholder nucleosomes, and the potential role of CENP-A at DNA damage sites. Elucidating the similarities and differences between these separated processes will further our understanding of the fundamental mechanisms controlling chromatin biology and promote new therapies against diseases arising from centromeric defects and altered CENP-A dynamics.

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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.

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