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REVIEW

Stable inheritance of CENP-A chromatin: Inner strength versus dynamic control

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Chromosome segregation during cell division is driven by mitotic spindle attachment to the centromere region on each chromosome. Centromeres form a protein scaffold defined by chromatin featuring CENP-A, a conserved histone H3 variant, in a manner largely independent of local DNA cis elements. CENP-A nucleosomes fulfill two essential criteria to epigenetically identify the centromere. They undergo self-templated duplication to reestablish centromeric chromatin following DNA replication. More importantly, CENP-A incorporated into centromeric chromatin is stably transmitted through consecutive cell division cycles. CENP-A nucleosomes have unique structural properties and binding partners that potentially explain their long lifetime in vivo. However, rather than a static building block, centromeric chromatin is dynamically regulated throughout the cell cycle, indicating that CENP-A stability is also controlled by external factors. We discuss recent insights and identify the outstanding questions on how dynamic control of the long-term stability of CENP-A ensures epigenetic centromere inheritance.

Introduction

The nucleosome core particle (NCP) is the basic unit of eukaryotic chromatin that packages genomic DNA within the nucleus (Kornberg, 1974). NCPs consist of 147 bp of DNA wrapped around a heterooctameric organization of pairs of the histone proteins H2A, H2B, H3, and H4 (Luger et al., 1997). Nucleosomal packaging controls access to nucleic acid binding proteins and is regulated by DNA methylation or histone modifications (Kouzarides, 2007). These constitute two major epigenetic features that contribute to tissue-specific gene expression patterns and global gene silencing. In addition to modification to DNA or histones, incorporation of variant histones impacts nucleosome structure, another key mechanism of chromatin regulation (Talbert and Henikoff, 2010). Histone variants index the genome into functional domains (Loyola and Almouzni, 2007). Major genome differentiation is imposed by H3 variants: H3.3 is enriched at transcriptionally active chromatin, while CENH3, more widely known as centromere protein A (CENP-A) in humans (Earnshaw and Rothfield, 1985), marks functional centromeres (Henikoff and Smith, 2015). In mitosis, the centromere forms the scaffold for the assembly of the kinetochore, a multiprotein complex that attaches to spindle microtubules to ensure sister chromatid segregation (McKinley and Cheeseman, 2016). Centromeres are a classic example of an epigenetically regulated chromatin locus. Early studies in dicentric human chromosomes revealed that centromeric DNA sequences are not sufficient to nucleate centromeric chromatin (Earnshaw and Migeon, 1985), suggesting that an independent trigger is required. Current evidence indicates that CENP-A constitutes this trigger in humans (Earnshaw and Migeon, 1985) or Cid in *Drosophila* (Henikoff et al., 2000), and Cse4 in budding yeast (Meluh et al., 1998). CENP-A is essential for localization of most of the inner and outer kinetochore proteins (Collins et al., 2005; Fachinetti et al., 2013). Critically, forced targeting of CENP-A to chromatin is sufficient to seed centromeres, and de novo centromeres are maintained epigenetically after removal of the initial targeting signal. Combined, these results provide strong evidence that CENP-A forms a key part of an epigenetic mark that identifies, maintains, and propagates centromeres (Mendiburo et al., 2011; Barnhart et al., 2011; Hori et al., 2013).

The central hypothesis that follows is that CENP-A-containing nucleosomes are present at all times to maintain centromere identity. In this review we ask, how stable is CENP-A at centromeres, and how is this stability achieved? The maintenance of nucleosomes in chromatin is threatened by several processes, most notably the disruptive forces of DNA replication, transcription, chromatin compaction, and the forces exerted during chromosome segregation. We focus on the mechanisms of maintenance of CENP-A–containing nucleosomes at human centromeres, with occasional examples from other model organisms having CENP-A–based centromeres, such as yeasts and *Drosophila*. In this context, it is relevant to

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mention that in certain phyla, such as Kinetoplastids and Lepidopteran insects, centromeres have evolved without a CENP-A orthologue. The kinetochore organization in these unique species is an active field of research and has been covered elsewhere (Drinnenberg and Akiyoshi, 2017).

Centromeric CENP-A is stably transmitted through cell division

Self-templated duplication and stable propagation through S-phase and mitosis are essential to maintain an epigenetic mark through the cell cycle. Temporally controlled pulse labeling of CENP-A has offered powerful insights into the rate and timing of CENP-A deposition and turnover. Experiments in *Drosophila* embryos and human cells revealed that new CENP-A is assembled into centromeres during mitotic exit (Jansen et al., 2007; Schuh et al., 2007). CENP-A deposition involves a dedicated machinery, including Holliday junction recognition protein (HJURP), a conserved CENP-A chaperone that recognizes and binds to a specific structural domain in CENP-A (Foltz et al., 2009; Dunleavy et al., 2009; Bassett et al., 2012). This has led to the view that de novo CENP-A deposition is self-templated and cell cycle restricted (reviewed extensively in McKinley and Cheeseman, 2016). From the punctuated intervals of CENP-A assembly, it follows that, once incorporated, CENP-A should be stable in chromatin to maintain centromere identity, at least until the next round of deposition. Indeed, pulse labeling of chromatin-bound CENP-A revealed little turnover (Hemmerich et al., 2008; Jansen et al., 2007). Specifically, in cycling HeLa cells, the half-life of centromeric CENP-A is ~20 h, equating to the average length of the cell cycle (Bodor et al., 2013). This indicates that, once incorporated into centromeres, CENP-A has essentially no turnover, except for undergoing replicative dilution during DNA replication and cell division (Fig. 1, A and B). This finding is significant, as other H3 variants turn over much faster (Bodor et al., 2013; Deaton et al., 2016). Furthermore, experiments based on pulse labeling coupling to cell-to-cell fusion demonstrated that chromatin-bound CENP-A does not exchange between centromeres, indicating maintenance in cis (Falk et al., 2015).

Transgenerational inheritance of centromeric chromatin

In addition to the long half-life in cycling somatic cells, CENP-A is retained in gametes of several (Palmer et al., 1990; Raychaudhuri et al., 2012; Dunleavy et al., 2009), although not all (Monen et al., 2005; Gassmann et al., 2012) animal species. CENP-A retention even in the male germline is significant, as histones are typically evicted en masse and replaced by protamines (Bošković and Torres-Padilla, 2013). Meiotic cell cycles pose additional challenges for the stable inheritance of CENP-A nucleosomes. Principally, during oogenesis, oocytes arrest at prophase I of meiosis I and can stay suspended at that stage for months in mice to decades in humans until fertilization occurs (MacLennan et al., 2015). A genetic cross resulting in a CENP-A–null allele in the mouse germline demonstrated that CENP-A, assembled at the centromeres before meiotic arrest, is retained for more than a year and can be observed in aged oocytes (Smoak et al., 2016; Fig. 1 C). While centromeric chromatin can be remarkably stable, an absolute lack of turnover leaves no room for regulation or accommodation of stochastic fluctuation. Recent work revisited CENP-A stability at longer timescales in human somatic cells that underwent long-term arrest induced by quiescence and found a turnover of 2–10% of centromeric CENP-A per day (Swartz et al., 2019). Similar observations were made in prophase 1–arrested starfish oocytes, in which CENP-A turnover appears driven by transcription-mediated eviction of parental nucleosomes (Swartz et al., 2019). This suggests that erosion of parental CENP-A nucleosomes can occur due to spurious transcription events at the centromere, which becomes evident at the long timescales of meiotic cell cycle arrest. In summary, these studies demonstrate that while CENP-A can be unusually long lived, whether it does so is subject to species-specific control and depends on the challenges faced by the local chromatin environment.

The question that follows is how is CENP-A stability achieved, and how can stable transmission be regulated? Next we focus on intrinsic properties of CENP-A to help explain its long-term transmission.

Intrinsic physical characteristics of CENP-A nucleosomes

CENP-A has a domain architecture similar to that of canonical H3, with a divergent N-terminal tail and a conserved histone fold domain (Fig. 2 A; Palmer et al., 1991; Sullivan et al., 1994). The CENP-A-H4 complex is more rigid compared with its H3–H4 counterpart, and CENP-A-H4 tetramers have a more compact structure (Black et al., 2004). Although the overall fold is conserved between CENP-A and H3, residues within the α 2 helix and loop 1 of CENP-A are more divergent (Fig. 2 C, inset). Replacing this region of H3 with the corresponding CENP-A residues resulted in centromere targeting of the chimeric protein (Black et al., 2004). Therefore, this CENP-A targeting domain (CATD) contains the structural features that target CENP-A to the centromeres. The CATD is also essential for binding to the CENP-A chaperone HJURP, both in solution and within the nucleosome (Dunleavy et al., 2009; Foltz et al., 2009; Zasadzińska et al., 2013). A crystal structure of the CENP-A-H4 tetramer using recombinant N-terminal truncated CENP-A further indicated that changes in loop 1 of CENP-A bring downstream residues of CENP-A closer to H4, thereby leading to a region of hydrophobic stitching that contributes to the CENP-A-H4 core rigidity (Sekulic et al., 2010; Fig. 2 D). The first crystal structure of the CENP-A nucleosome was solved at 3.6-Å resolution using recombinant histones (Tachiwana et al., 2011; Fig. 2 C), revealing an octameric structure with the DNA wrapped around the nucleosome in a lefthanded manner. Despite possible variations in composition (Dalal et al., 2007; Dimitriadis et al., 2010; Mizuguchi et al., 2007; Bui et al., 2012), converging in vivo data support the conventional octameric arrangement (Hasson et al., 2013; Nechemia-Arbely et al., 2017) of CENP-A nucleosomes, with recent estimates of ~80% of homotypic CENP-A octamers on centromeric α-satellite DNA across all phases of the cell cycle (Nechemia-Arbely et al., 2017).

Divergent DNA wrapping by CENP-A nucleosomes

A key feature revealed by the atomic model of CENP-A nucleosomes is the unwrapping of ~13 bp of DNA at each end of the

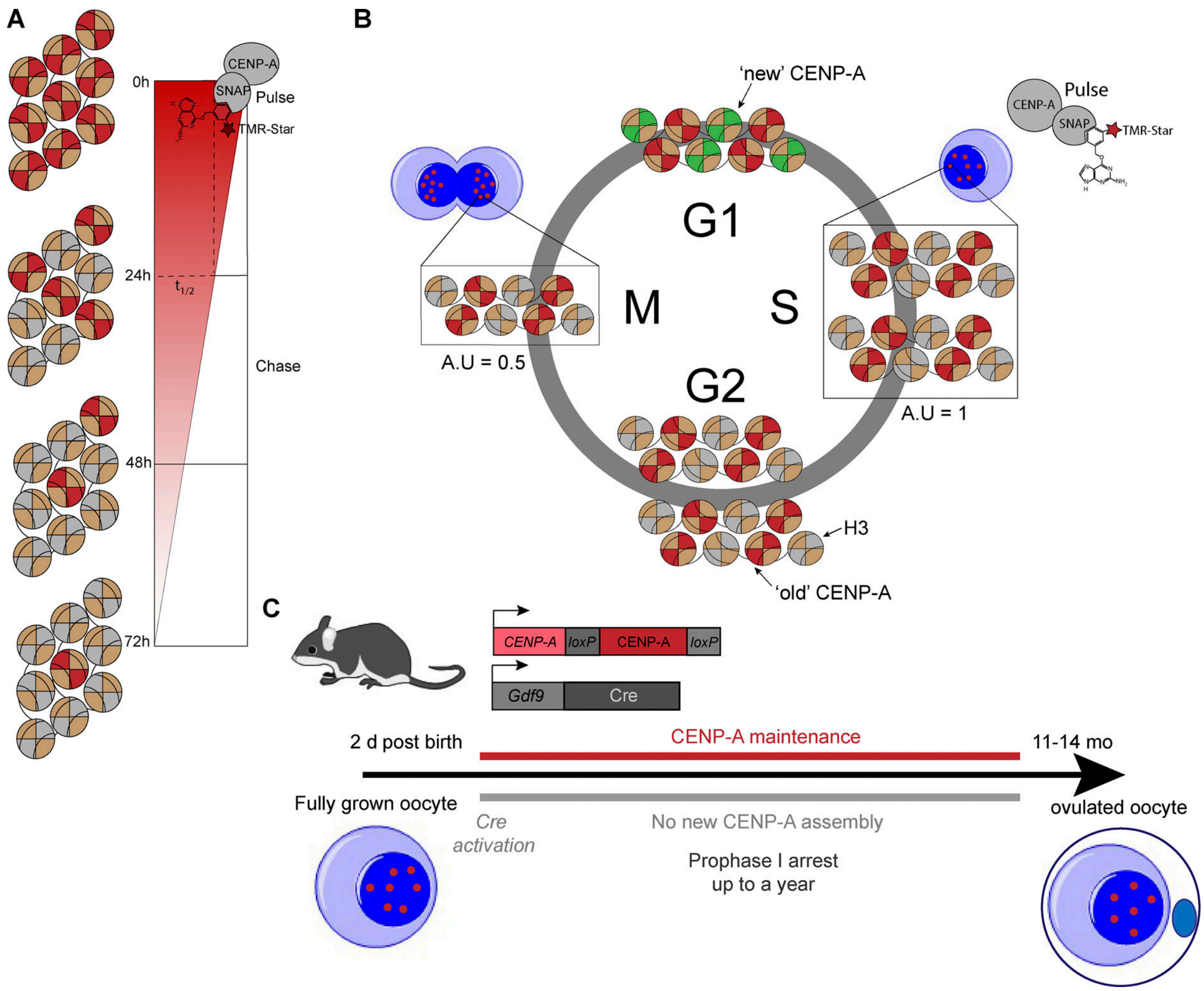

Figure 1. **Stable inheritance of CENP-A at centromeres through cell divisions as well as transgenerationally. (A)** Schematic depicting pulse-chase assay based on CENP-A-SNAP labeled with a fluorescent dye (TMR-Star). Quantification of CENP-A-SNAP intensity through time reveals a half-life equal to that of the cell cycle, indicating quantitative inheritance. **(B)** Schematic of cell cycle–coupled dilution of centromeric chromatin determined by CENP-A-SNAP pulse-chase assay. A.U, arbitrary units. **(C)** Experimental setup to determine dynamics of preincorporated CENP-A through meiotic arrest in mice. Endogenous CENP-A coding region is flanked by loxP sites. The Cre recombinase is expressed under *Gdf9* promoter, which is active only in early stage oocytes at birth. Cre-mediated excision of CENP-A at this stage ensures that there is no further CENP-A assembly until oocyte maturation at 11–14 mo. CENP-A measurements in fully grown oocyte at birth and ovulated oocytes after 11–14 mo shows retention of CENP-A (red dots), which was assembled before birth.

CENP-A nucleosome due to a shorter αN helix of CENP-A (Fig. 2 B; Tachiwana et al., 2011; Ali-Ahmad et al., 2019). Replacing the N-terminal end along with the CATD domain of H3 with those of CENP-A is sufficient to assemble the typical CENP-A octameric nucleosome with such unwrapped DNA termini (Nechemia-Arbely et al., 2017), indicating the importance of the CENP-A N-terminal residues. The possible functional importance of these flexible DNA ends was borne out in recent cryo-EM studies, resulting in CENP-A nucleosomes adopting different nucleosome packing when embedded into an array of H3 nucleosomes (Takizawa et al., 2020).

However, to what extent the DNA entry and exit paths impact the conformation of CENP-A nucleosomes in vivo may be dictated by the local chromatin context. MNase footprinting showed that CENP-A nucleosomes have a smaller footprint, consistent with in vitro data (Hasson et al., 2013). However, different subpopulations of CENP-A exist (as measured by salt solubility). Loosely bound CENP-A tends to have the characteristic narrow footprint due to its reduced DNA wrapping, whereas CENP-A in complex with other centromere components forms very stable structures that encompass larger DNA fragments (Thakur and Henikoff, 2018, 2016). Indeed, structural studies in yeast showed that the unwrapped DNA termini are contacted by CENP-N (elaborated further in the next section; Yan et al., 2019). This raises the intriguing possibility that unwrapping of DNA ends may be key to the function of CENP-A nucleosomes, not only to impact higher-order wrapping through flexibility but to form a point of contact within the centromere complex.

The sufficiency of various domains of CENP-A in maintaining a sustained centromere function was tested in an elegant in vivo system using conditional Cre recombinase-mediated inactivation of endogenous CENP-A gene, rescued with H3 chimeras of different CENP-A subdomains (Fachinetti et al., 2013). Although

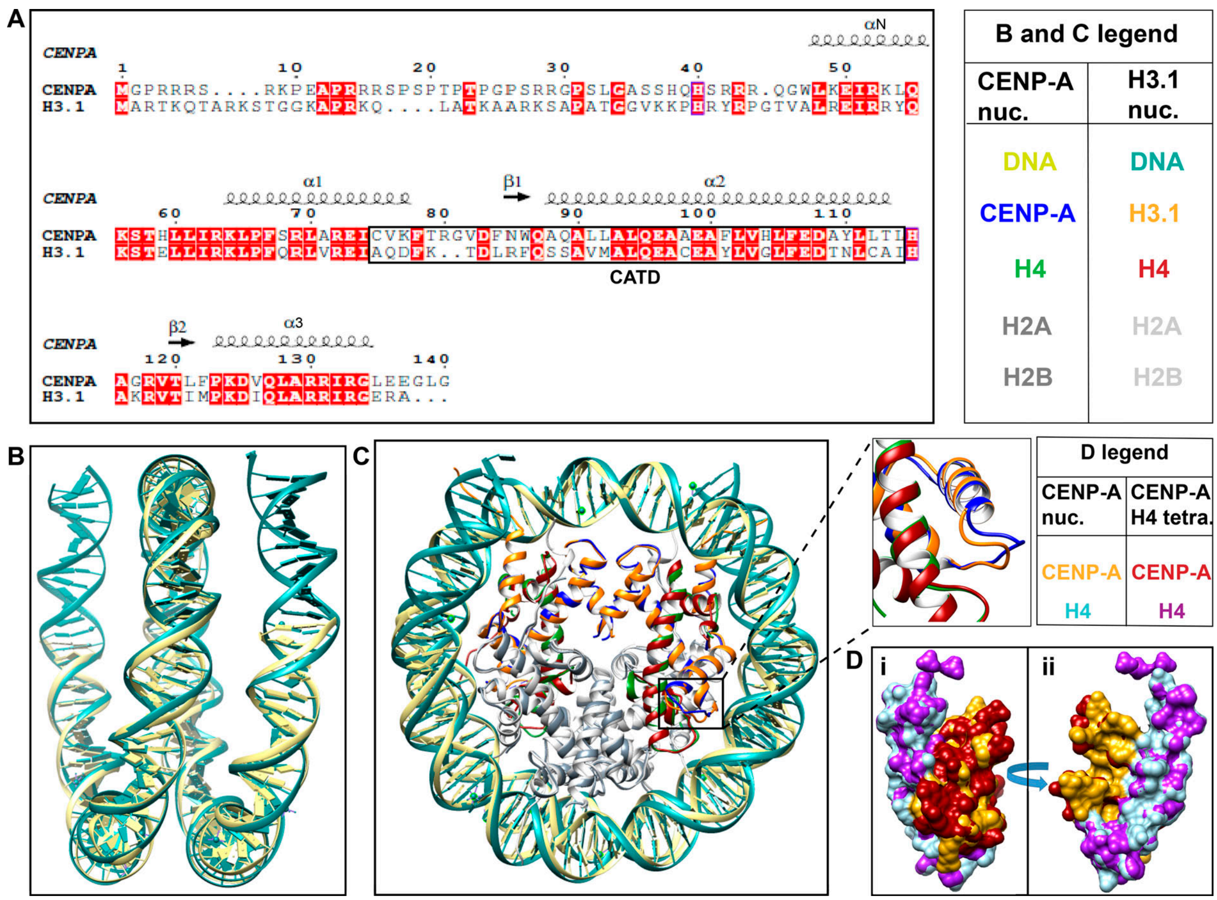

Figure 2. **Physical characteristics of the CENP-A nucleosome. (A)** Pairwise alignment of human CENP-A (NCBI Protein accession no. NP_001800.1) and H3.1 (NCBI Protein accession no. NP_003522.1) sequences, showing the secondary structural features of CENP-A. Residues that are 100% conserved are shown in red. **(B)** Overlay of nucleic acid sequences structured within the CENP-A nucleosome crystal (derived from PDB accession no. 3AN2) with those from the H3.1 nucleosome structure (PDB accession no. 5Y0C), respectively, showing loss of structured DNA termini, indicating unwrapping, in the CENP-A nucleosome. **(C)** Structural superposition of the complete CENP-A (PDB accession no. 3AN2) and histone H3.1 nucleosome (PDB accession no. 5Y0C). The inset shows a zoomed view of the loop 1 distension in the CENP-A nucleosome vis-à-vis the H3 nucleosome. **(D)** Comparison of CENP-A:H4 as heterotetramer (PDB accession no. 3NQJ) compared with CENP-A:H4 in nucleosomes (PDB accession no. 3AN2), with special emphasis on hydrophobic stitching. Representation is rotated 180° to show two opposite surfaces: i, nucleosome surface; and ii, nucleosome interior. CENP-A-H4 in the tetramer structure as well as nucleosomal CENP-A-H4 show a similar occupancy when viewed on the nucleosome surface (i). However, viewed from the interior (ii), nucleosomal CENP-A-H4 is predominantly seen, indicating that CENP-A-H4 derived from the heterotetramer is buried due to hydrophobic stitching.

the CATD domain was sufficient for maintaining the epigenetic self-templated duplication, the inclusion of the N- or C-terminal tail of CENP-A functionally rescued kinetochore assembly, allowing long-term maintenance of centromeric chromatin. Therefore, the unique structural properties of CENP-A that modify the shape of the nucleosome and the DNA wrapped around it not only facilitate its recognition by centromere proteins but also enhance its stable retention through the cell cycle. In the next section, we discuss how CENP-A nucleosome binding proteins contribute to maintaining the stability of CENP-A in vivo.

The role of CENP-A interacting proteins in stabilizing CENP-A nucleosomes

We have discussed how intrinsic structural features of CENP-A nucleosomes may contribute to their stable retention in chromatin. However, in vivo pulse labeling experiments and genomics approaches have shown that such retention is restricted to centromeres, as CENP-A at other genomic locations is rapidly depleted with a half-life similar to that of canonical histones (Falk et al., 2015; Nechemia-Arbely et al., 2019). Therefore, while nucleosomal CENP-A may have intrinsic properties that render it stable in chromatin, additional centromere-specific features are important contributors to the high stability. CENP-A nucleosome-containing chromatin recruits a heterooligomeric complex of 16 proteins named the constitutive centromere-associated network (CCAN), which remains associated with the centromere throughout the cell cycle. This group of proteins is classified into five subgroups based on immuno-pulldown and native size fractionation, namely centromere protein C (CENP-C), -LN, -HIKM, -TWSX, and -OPQUR subcomplexes (Weir et al., 2016; Hori et al., 2008; Foltz et al., 2006; Okada et al., 2006; Izuta et al., 2006; Obuse et al., 2004; Fig. 3, Bi, Bii, and Biii). The CCAN forms a structural scaffold for the assembly of the kinetochore through the recruitment of the KMN (KMN1, Mis12, and Ndc80 complexes) network, which binds to the microtubules during

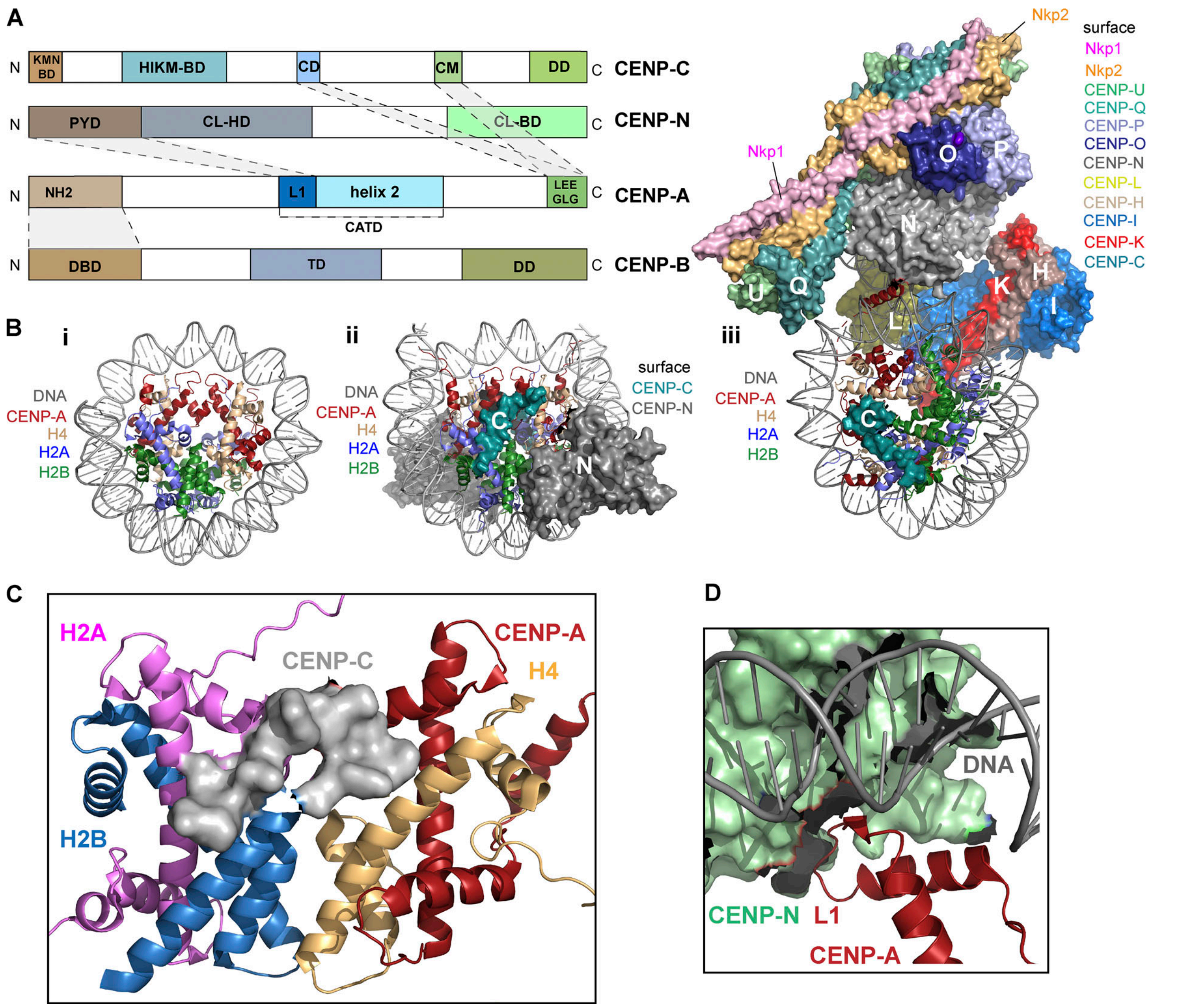

Figure 3. **Interactions of CENP-A with CCAN complex. (A)** Diagram depicting the domain organization of CENP-A and key CENP-A binding proteins CENP-B, -C, and -N. KMN-BD, Knl1-Mis12-Ndc80-binding domain; HIKM-BD, CENP-HIKM binding domain; DD, dimerization domain; PYD, PYRIN domain; CL-HD, CENP-L homology domain; CL-BD, CENP-L binding domain; NH2, N-terminal tail; L1, loop 1; DBD, DNA binding domain; TD, transposase-like domain. Pairwise interactions are indicated by dotted lines. **(B)** Hierarchy of CCAN organization with representative structures at three levels: i, CENP-A nucleosome (PDB accession no. 3AN2); ii, CCNC (PDB accession no. 6MUP); and iii, yeast CCAN complexed with CENP-A nucleosome (PDB accession no. 6QLD). All CCAN proteins are shown in surface representation, and CENP proteins are labeled in the structure with their unique letter designation only for brevity. **(C)** Detail of CENP-C CD contacts within CENP-A nucleosome (PDB accession no. 6SE6). **(D)** Detail of CENP-N contacts within CENP-A nucleosome (PDB accession no. 6COW). L1, loop 1 of CENP-A.

mitosis (Cheeseman, 2014). Three principle CENP-A binding proteins are known: CENP-C, -N, and -B. We explore the possibility that these proteins contribute to CENP-A nucleosome stability in vivo.

CENP-C

CENP-C is a large modular protein, with homologues present in all major model organisms. The N-terminal region contains domains binding to the Mis12 complex of the kinetochore and the CENP-HIKM complex (Fig. 3 A; Klare et al., 2015). CENP-C also contains a central domain (CD), conserved in mammals, that was found to directly bind to CENP-A nucleosomes (Carroll et al., 2010). The C-terminal region contains another CENP-A binding domain termed the CENP-C motif (CM) which is conserved across major eukaryotic lineages (Cohen et al., 2008), followed by the CENP-C dimerization domain at the extreme C-terminal end. In vitro binding assays and structural studies showed that both CENP-C CM and CD bind to a LEEGLG motif at the extreme C-terminal end of CENP-A as well as an acidic patch formed by H2A and H2B on the surface of the CENP-A nucleosomes (Fig. 3 C; Guo et al., 2017; Ali-Ahmad et al., 2019; Allu et al., 2019; Kato et al., 2013; Carroll et al., 2010; Guse et al., 2011). In vitro competition assays between CENP-C CD and CM indicate that for mammalian CENP-A nucleosomes, CENP-C CD forms the major CENP-A binding domain (Ali-Ahmad et al., 2019). Such mutually exclusive binding domains may indicate regulatory roles for alternative modes of CENP-C binding, as observed in chicken CENP-C (Nagpal et al., 2015). Alternatively, it may facilitate internucleosomal contacts. Importantly, CENP-C not only binds CENP-A but reshapes it, resulting in nucleosome compaction, possibly increasing stability (Falk et al., 2015, 2016). These in vitro studies were borne out by in vivo by pulse-chase labeling of CENP-A coupled to CENP-C depletion, demonstrating that CENP-C is required for CENP-A retention at the centromeres in vivo (Mitra et al., 2020; Falk et al., 2015; Guo et al., 2017). Consistent with a dominant role in vitro (Ali-Ahmad et al., 2019) the CENP-C CD was found to be critical to stabilize CENP-A in vivo by both direct interactions with CENP-A and contacts with H2A of the CENP-A nucleosome (Guo et al., 2017; Fig. 3 C).

CENP-N

CENP-N was the first protein found to directly bind to CENP-A nucleosomes in in vitro nucleosome binding assays (Carroll et al., 2009). Structural studies revealed that CENP-N interacts primarily through its N-terminal PYRIN domain to the L1 loop of the CENP-A CATD domain (Fig. 3, A and D; Pentakota et al., 2017; Chittori et al., 2018). Additionally, it makes several contacts to nucleosomal DNA stabilizing CENP-A DNA interactions (Pentakota et al., 2017; Guo et al., 2017). Reconstitution of a combined CENP-A^{Nuc}/-C/-N core centromeric nucleosome complex (CCNC) revealed a stoichiometry of one or two copies of the CENP-N N-terminus and two copies of CENP-C CD bound to the CENP-A NCP (Allu et al., 2019; Fig. 3 Bii). Finally, pulse labeling assays coupled to acute depletion of CENP-N showed that CENP-N along with CENP-C is required for CENP-A retention in vivo (Guo et al., 2017), although this finding has been nuanced (Cao et al., 2018), indicating that the degree to which CENP-C and -N contribute to CENP-A stability in vivo is yet to be clearly established.

CENP-B

CENP-B is a centromeric protein known to bind specific sequence motifs, called CENP-B boxes, enriched within α-satellite sequences that are common at centromeres (Masumoto et al., 1989; Gamba and Fachinetti, 2020). While CENP-B is nonessential for centromere function in vivo (Hudson et al., 1998), nucleosome binding assays found that in addition to binding DNA, CENP-B directly interacts with the N-terminal end of CENP-A (Fujita et al., 2015; Fachinetti et al., 2015; Fig. 3 A). CENP-B was also found to indirectly contribute to CENP-A stability by interacting directly with CENP-C and contributing to its centromeric maintenance (Fachinetti et al., 2015).

A cooperative assembly of centromere proteins contributing to CENP-A nucleosome stability

Apart from the individual interactions, different studies indicate that the CCAN-mediated stabilization of CENP-A nucleosomes may involve multivalent interactions with one or more subcomplexes of the CCAN complex. For example, biochemical reconstitution of human kinetochores identified that the CENP-CHIKMLN 7-unit subcomplex of the CCAN has the highest affinity to CENP-A nucleosomes, compared with CENP-C and -LN subcomplexes alone (Weir et al., 2016), indicating cooperative binding. This observation was supported in vivo by inducible CRISPR/Cas9-mediated knockouts of individual CCAN subunits. CENP-C knockout led to loss of centromeric localization of all other CCAN proteins at all stages of the cell cycle (McKinley et al., 2015). Conversely, the interphase localization of CENP-C was stabilized by CENP-HIKM and -LN complexes. Further CENP-I, -N, and -T (McKinley et al., 2015) and Mis12 (Kline et al., 2006) depletion led to a modest decrease in CENP-A levels from the centromere. Finally, a recent cryo-EM structure of the budding yeast CCAN complexed with the CENP-A nucleosome (Yan et al., 2019) revealed a structure in which the CENP-A nucleosome is centrally located with the CENP-HIK head domain and the CENP-QU subunits interacting with CENP-A and the nucleosomal DNA gyre at opposite ends (Fig. 3 Biii). Combined, this indicates that a series of contacts are made to ensure cooperative CCAN and CENP-A nucleosome stability. Our discussion thus far suggests that centromere complex assembly is a major contributor to stabilizing centromeric chromatin. However, in cycling cells, the centromere complex faces the challenge of the disruptive forces of DNA replication and transcription machineries, as well as physical forces exerted during cell division. Next we aim to understand how CENP-A nucleosomes navigate these challenges.

DNA replication and chromatin maintenance

Genome duplication is one of the principle challenges in the preservation of chromatin structure. During DNA replication, parental nucleosomes are known to be disassembled ahead of the replication fork into tetramers disrupting chromatin structure. These are subsequently reassembled onto daughter DNA strands along with newly deposited histones in a 1:1 stoichiometry (Xu et al., 2010; Alabert et al., 2015; Fig. 4 B). Accurate recycling of parental histones is essential for maintaining the positional signature of histones that are locally decorated with posttranslational modifications to establish transcriptionally competent or repressive domains (Bannister and Kouzarides, 2011). Moreover, in some cases, modifications impact histone stability, such as the faster turnover of acetylated H3 (Zee et al., 2010). Genome-wide chromatin immunoprecipitation sequencing of nascent DNA for active as well as repressed chromatin revealed that the parental epigenetic landscape remains preserved in the newly replicated DNA, indicating a local and accurate recycling of parental histones along with their modifications (Reverón-Gómez et al., 2018).

This maintenance of positional memory was found to be dependent on the replicative helicase mini-chromosome maintenance 2 (MCM2) in mouse (Petryk et al., 2018) and the leading strand DNA polymerase subunits DNA polymerase ε III and IV (DPB3 and 4) in yeast (Yu et al., 2018). MCM2 acts in a complex with H3–H4 (Groth et al., 2007), where the N-terminal histone-binding domain (HBD; aa 61–130) selectively binds H3–H4 with the stoichiometry of two MCM2 HBDs binding to a single H3–H4 tetramer (Huang et al., 2015). Structural work indicates MCM2 HBD binding laterally to the H3–H4 dimer (Fig. 4 A), occluding the DNA binding surface of H3–H4 as well as the interaction surface between H4 and H2B in the nucleosome. The histone chaperone anti-silencing factor 1 (ASF1) has been proposed to interact with MCM2-H3/H4 to recycle histones (Groth et al., 2007). A crystal structure of the combined MCM2

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(HBD)-H3–H4-ASF1 complex showed that ASF1 primarily interacts with a H3–H4 dimer in the presence of MCM2, thereby indicating a structural transition from tetrameric H3–H4 bound to MCM2 to a H3–H4 dimer bound by both MCM2 and ASF1 (Huang et al., 2015; Fig. 4 E). Based on pulse labeling experiments, MCM2 binding was found to be dispensable for replication-coupled new nucleosome assembly but required for the overall stability of H3–H4 (Huang et al., 2015). Further, upon decoupling of the MCM2-7 replication helicase from DNA polymerase, ASF1 was found in intermediate complexes, which also consisted of MCM2 and parental H3–H4 (Groth et al., 2007). These observations support a model in which ASF1 cochaperones with MCM2 to transfer parental histones to newly synthesized DNA strands (Fig. 4 B). How do the canonical mechanisms of chromatin replication at the replication fork apply to the inheritance of CENP-A nucleosomes?

The role of replication in recycling CENP-A

The problem of histone maintenance becomes more acute for CENP-A nucleosomes, whose loading occurs only following the next mitosis. This means that during DNA replication, loss of CENP-A nucleosomes cannot be immediately compensated for with de novo assembly. The observation that ASF1 cochaperones with MCM2 to transfer parental histones to newly synthesized DNA strands raises the question as to whether a similar mechanism underlies recycling of CENP-A. Comparison of the CENP-A-H4 tetramer in the CENP-A nucleosome with the structure of the MCM2-H3-H4 tetramer indicates that the region of H3 involved in interaction with MCM2 is conserved in CENP-A (Zasadzińska et al., 2018; Huang et al., 2015; Fig. 4 C). Supporting this, in vitro assays showed that CENP-A is robustly bound to MCM2 HBD, and the binding is abolished by the MCM2 double mutant (Y81A, Y90A) that abrogates the histone binding ability of MCM2. Further, the CENP-A chaperone HJURP interacts with MCM2 in vitro in a histone-independent manner (Huang et al., 2015). The role of HJURP in chaperoning parental CENP-A nucleosomes was consolidated in a study that used proximity-based in vivo labeling (BioID) to directly identify proteins that are transiently, yet specifically, associated with CENP-A nucleosomes during S phase (Zasadzińska et al., 2018). HJURP was found to be specifically enriched with CENP-A nucleosomes during replication. Fluorescent pulse-chase assays coupled to acute degradation of HJURP showed a loss in retention of preassembled CENP-A, indicating that HJURP is required for recycling of parental CENP-A nucleosomes. The R63 and K64 residues of H3 had been identified previously as residues that are important for binding to MCM2 (Huang et al., 2015). These residues are conserved in CENP-A, and their mutation results in a modest reduction in binding to purified MCM2 HBD in vitro. Concomitantly, these mutants also showed enhanced loss from the centromere during S phase. Finally, coimmunoprecipitation assays showed that endogenous MCM2 interacts with HJURP in vivo, indicative of a similar role for HJURP and ASF1 in chaperoning their respective partner nucleosomes (Zasadzińska et al., 2018; Huang et al., 2015; Figs. 4 D and 5 C). However, the mode of interaction of HJURP to the CENP-A nucleosome is distinct from that shown for ASF1 for the H3.3 nucleosome. While the HJURP interaction surface extends all along the α 2 helix of CENP-A, ASF1 interacts primarily with the α 3 helix of H3.3 (Fig. 4 E; Hu et al., 2011; Huang et al., 2015). As a direct extension of this observation, it is posited that ASF1 or another replication coupled histone chaperone collaborates with HJURP to cochaperone with MCM2. The apparent modest role for HJURP in recycling CENP-A also indicates that other chromatin remodelers might be involved. Identifying such factors will be a key step forward in understand how CENP-A stability is transmitted during replication.

Efficient recycling of CENP-A during DNA replication is restricted to the centromere

Another indication that parental CENP-A nucleosomes are locally retained was revealed by CENP-A chromatin immunoprecipitation after cell cycle synchronization. Mapping of the enriched sequences to annotated human centromeric DNA models revealed that the position of CENP-A nucleosomes is conserved in G1 and G2 centromeres, indicating that the initial centromeric loading sites are maintained through replication (Nechemia-Arbely et al., 2019). Several studies have indicated that a significant fraction of CENP-A is loaded into noncentromeric sites (Bodor et al., 2014; Lacoste et al., 2014; Nye et al., 2018), in part as H3.3/CENP-A heterotypic nucleosomes involving the H3.3 chaperone DAXX (Arimura et al., 2014; Lacoste et al., 2014). Such H3.3-containing nucleosomes may help explain why CENP-A outside the centromere is more dynamic (Falk et al., 2015). Strikingly, the cell cycle–specific analysis of CENP-A occupancy revealed that the noncentromeric pool is selectively removed during passage through S-phase, whereas centromeres quantitatively retained CENP-A (Fig. 4 F; Nechemia-Arbely et al., 2019). A similar observation was made in fission yeast, where newly deposited CENP-A was found to be transiently incorporated in chromosome arms before being rapidly removed during S phase (Shukla et al., 2018). Affinity purification of CENP-A nucleosomes in late S phase, when mammalian centromeric DNA replicates, followed by mass spectrometry revealed that the entire 16-subunit CCAN complex remains associated with CENP-A mononucleosomes (Nechemia-Arbely et al., 2019). This suggests that, as discussed above, the CCAN may help tether CENP-A nucleosomes in place, enabling their retention during the passage of the replication fork. Indeed, acute auxin-mediated depletion of CENP-C during late S phase resulted in a 73% loss of CENP-A enrichment from centromeres (Nechemia-Arbely et al., 2019). Further, robust association of MCM2 to CENP-A nucleosomes was observed only in late S phase (Nechemia-Arbely et al., 2019). Taken together, these results indicate a key role of CENP-C and/or CCAN to stabilize parental CENP-A nucleosomes during replication to facilitate their recycling by HJURP-MCM2 or other chaperone complexes. A key question going forward is how is CCAN binding coordinates with replication-specific chaperones to ensure efficient CENP-A recycling specifically at the centromere.

The role of transcription in CENP-A stability

In addition to replication, transcription has the potential to disrupt nucleosome contacts, and contrasting roles have been described for transcription in centromere specification.

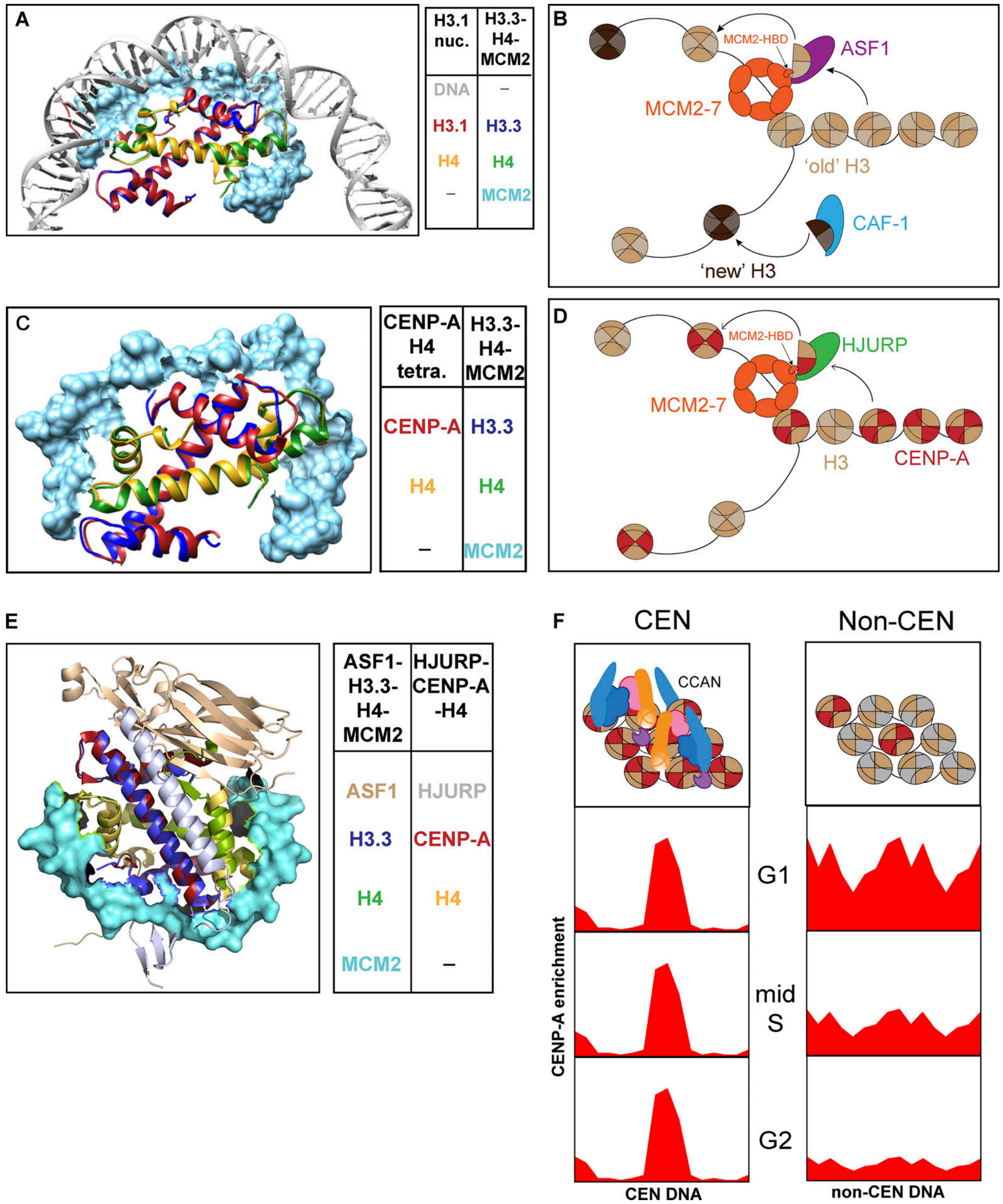

Figure 4. **Role of replication in chaperoning parental CENP-A nucleosomes. (A)** Superposition of H3.1, H4, and DNA from H3.1 nucleosome (PDB accession no. 5Y0C) with MCM2 HBD –H3.3-H4 tetramer (PDB accession no. 5BNV) showing the overlap of MCM2 interacting surface with DNA. **(B)** Model of HJURP- and ASF1-mediated chaperoning of parental H3 nucleosomes at the replication fork simultaneously with CAF-1–mediated deposition of new H3. **(C)** Superposition of CENP-A/H4 tetramer (PDB accession no. 3NQJ) with MCM2-HBD-H3.3/H4 tetramer (PDB accession no. 5BNV) showing conservation of the MCM2 interacting surface between CENP-A-H4 and H3–H4. **(D)** Model of HJURP- and MCM2-mediated chaperoning of parental CENP-A nucleosomes at the replication fork. **(E)** Superposition of MCM2-H3–H4-ASF1 (PDB accession no. 5BNX) and HJURP-CENP-A-H4 (PDB accession no. 3R45) structures showing the distinct modes of binding of ASF1 and HJURP chaperones to their respective histones in the context of cochaperoning with MCM2. **(F)** Schematic showing DNA replication–mediated removal of ectopic CENP-A. Left panel depicts the centromeric (CEN) chromatin with constitutively bound CCAN. Graphs represent the enrichment of CENP-A at native centromeres, which remains unchanged from G1 through S to G2. Right panel depicts CENP-A ectopically loaded (non-CEN) in euchromatin in G1. Graphs represent the ectopic enrichment of CENP-A at euchromatin in G1, which is subsequently removed during S phase. Bottom panels are schematic interpretations of CENP-A occupancy data reported in Nechemia-Arbely et al. (2019).

Traditionally, centromeres were thought to be transcriptionally silent, since they are predominantly embedded in heterochromatin and evolutionarily new centromeres are found at gene-poor regions (Cardone et al., 2006). Overexpression of centromeric transcripts or driving transcription through centromeres by a strong promoter dislodges the CENP-A chromatin, resulting in loss of centromere function (Hill and Bloom, 1987; Talbert and Henikoff, 2018; Nakano et al., 2008). However, recent results indicate that a low level of transcription is essential for centromere function by promoting and/or stabilizing CENP-A deposition (Bergmann et al., 2011; Catania et al., 2015; Chan et al., 2012). The transcription-associated chaperone FACT (facilitates

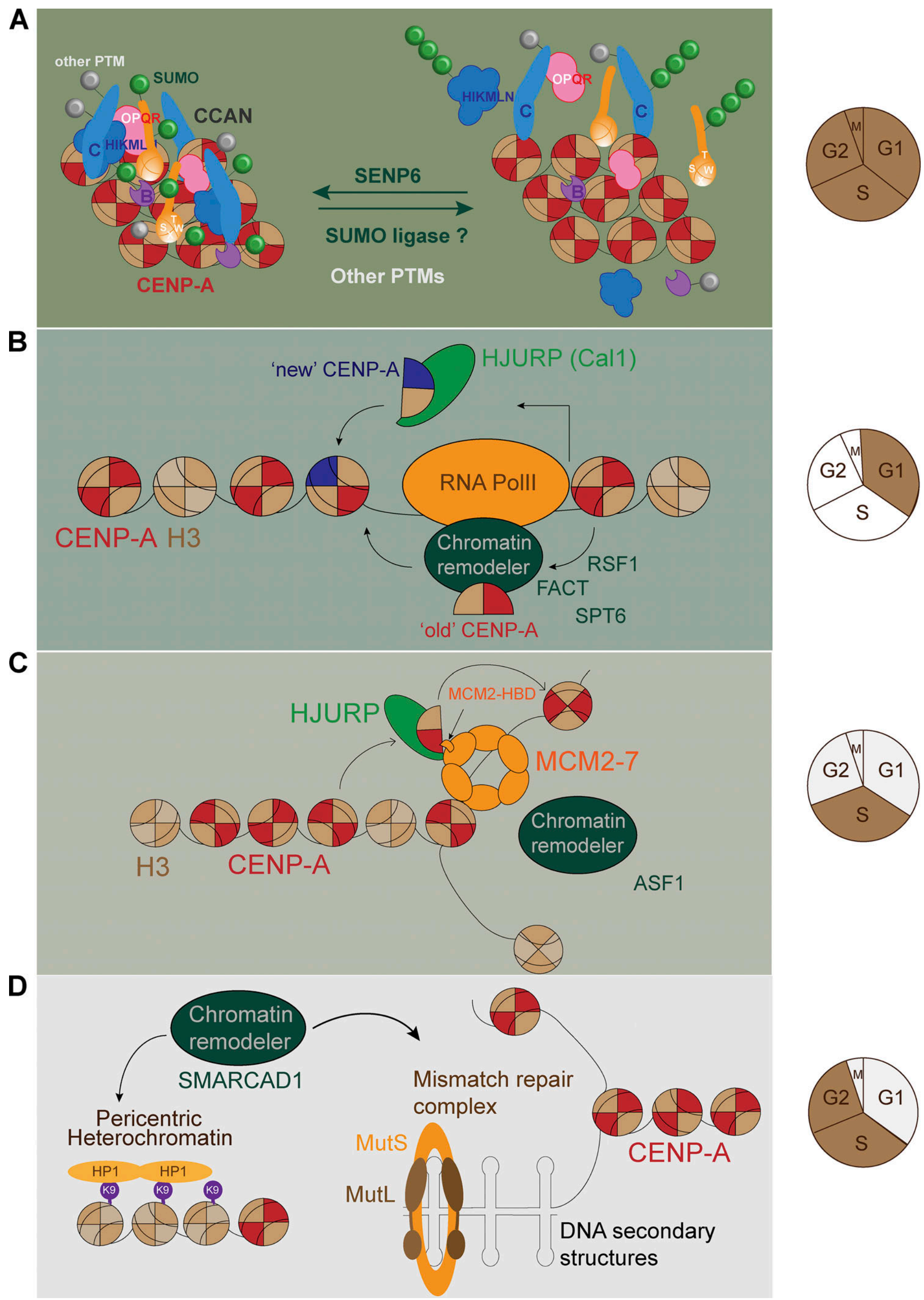

Figure 5. **Putative hierarchy of key processes that regulate CENP-A maintenance at the centromeres.** Left panels show the chromatin regulatory processes involved in CENP-A maintenance. Right panels indicate the cell cycle phases during which these processes are active. **(A)** CCAN and its dynamic modification by SUMOylation and other posttranslational modifications play a dominant role in centromeric maintenance of parental CENP-A. **(B)** RNA Pol II–mediated transcription and its presumptive role in CENP-A deposition and retention of parental CENP-A with the help of chaperones and remodelers as shown. **(C)** Parental CENP-A transfer reaction during DNA replication with key roles for MCM2 and HJURP. **(D)** Possible roles of pericentric heterochromatin maintenance and DNA mismatch repair system in regulating CENP-A nucleosome stability.

chromatin transcription) localizes to centromeres in chicken cells in a CENP-H–dependent manner. Further, conditional mutation of SSRP1, a FACT subunit, resulted in reduced CENP-A assembly at the centromere (Okada et al., 2009). In *Drosophila*, the CENP-A chaperone Cal1 (analogous to HJURP in mammals) was shown to bind FACT in a prenucleosomal complex (Chen et al., 2015). FACT is required for loading of new CENP-A, and its depletion led to the accumulation of H3.1 and H3.3 in the endogenous centromeric chromatin. These observations indicates a model in which Cal1-driven FACT localization remodels the centromeric chromatin, leading to the eviction of H3 nucleosomes and facilitating and/or stabilizing the deposition of CENP-A nucleosomes. RNA Pol II recruitment coupled to H3 eviction was also observed in centromeric core sequences in fission yeast (Shukla et al., 2018). The role of transcription in stable CENP-A incorporation was further underscored in a recent study in *Drosophila*, in which newly deposited CENP-A was found to exist in two distinct populations: a salt-sensitive pool that corresponded to newly recruited CENP-A, bound to its chaperone Cal1; and a stable salt-resistant pool whose incorporation depends on transcription and the FACT complex (Bobkov et al., 2018). However, while the disruption of histone octamers by FACT may facilitate CENP-A assembly, it may also disrupt preexisting CENP-A nucleosomes. The transcription elongation factor suppressor of Ty 6 (Spt6) is known to prevent transcription-coupled nucleosomal loss by reincorporating H3/H4 that have been displaced during transcription (Duina, 2011). Using recombination-induced tag exchange to track CENP-A dynamics, Spt6 was found to be required for maintenance of preincorporated CENP-A pool during the cell cycle window when new CENP-A is incorporated in *Drosophila* cells (Bobkov et al., 2020).

From these data, what emerges is that transcription is acting as a double-edged sword. On the one hand, it is required to drive chromatin turnover to allow new CENP-A assembly; such turnover, by the same token, threatens existing CENP-A nucleosomes. These contributing and detrimental roles of transcription may explain why its abundance and rate at the centromere need to be exquisitely titrated. How this is controlled remains an open question.

In addition to the remodeling force of the RNA polymerase, the transcript produced from centromeric DNA has been implicated in the maintenance of CENP-A in many species, including fission yeast (Choi et al., 2011), maize (Topp et al., 2004), mouse (Ferri et al., 2009), and humans (Wong et al., 2007). For instance, in maize, 40–200-ribonucleotide-long RNAs were found to be associated with CENP-A, -B, and -C (Topp et al., 2004). Similar results were also observed in mammalian cells, in which all human centromeric α-satellite repeats were found to produce transcripts that remained localized in cis (McNulty et al., 2017). These transcripts appeared stable and associated with chromatin-bound CENP-A, -B, and -C. Moreover, targeted depletion of α-satellite transcripts led to 30 and 44% reduction in array-specific CENP-A and -C proteins, indicating that RNA binding stabilizes one or more components of the CCNC. However, a recent report did not find evidence for a direct role for centromeric transcripts in stabilizing centromere components in cis, and instead suggested that centromere transcription possibly regulates the centromere–nucleolus association (Bury et al., 2020). In sum, the primary role of transcription appears to be as a motor to remodel chromatin to facilitate assembly and/or stabilization of newly loaded CENP-A, in conjunction with transcription-associated histone chaperones, FACT and Spt6 (Fig. 5 B). In addition, transcription may play a secondary role via centromeric transcript-mediated stabilization of CENP-A and -C. However, definitively assigning a selective role to RNA itself is challenging, as manipulation of it may have indirect effects on the transcription machinery. More synthetic approaches are needed to disentangle the role of RNA and the polymerase that produces it.

Protection of CENP-A chromatin during mitosis

A final cell cycle challenge to the maintenance of chromatin in general and CENP-A in particular is the transition through mitosis. Chromatin is condensed into mitotic chromatin and is subjected to microtubule pulling forces, particularly at the centromere. The extent of these forces become evident, for instance, during condensin depletion, which leads to a dramatic loss of centromere integrity (Ribeiro et al., 2009; Samoshkin et al., 2009). Recent work has shown a similar effect upon loss of Polo-like kinase 1 (Plk1), a major mitotic kinase that has multiple roles at the kinetochore (Lera et al., 2016). Inhibition of Plk1 leads to lagging chromosomes as cells progress toward anaphase (Lera and Burkard, 2012). These lagging chromosomes contain ruptured centromeric chromatin, resulting in loss of CENP-A, -C, and -T (Lera et al., 2019). This correlated with enhanced recruitment of Plk1-interacting checkpoint helicase (PICH) and the Bloom's syndrome protein helicase (BLM), which caused exaggerated unwinding of CENP-A chromatin. Thus, Plk1 maintains the integrity of CENP-A chromatin during mitosis by preventing excessive chromatin unwinding due to the high tension generated by the spindle microtubules. These findings show that physical mitotic forces may suffice in disrupting chromatin and that maintaining chromatin integrity is important for CENP-A transmission.

Ubiquitin control of CENP-A protein stability

Our discussion thus far has focused on aspects of the cell cycle that disrupt CENP-A chromatin and threaten stable transmission. However, emerging evidence indicates that CENP-A stability may be deliberately regulated and is subject to dynamic control. An early insight came from human cancers in which CENP-A is overexpressed (Sun et al., 2016) possibly contributing to chromosomal instability (Shrestha et al., 2017). These observations indicate that CENP-A levels must be regulated stringently at the RNA and protein levels. For instance, overexpressed CENP-A in budding yeast is polyubiquitinated specifically by the RING finger E3 ubiquitin ligase Psh1 (Pob3/Spt16 histone-associated 1) and targeted for degradation (Ranjitkar et al., 2010; Hewawasam et al., 2010; Collins et al., 2004). In the absence of Psh1, overexpressed CENP-A mislocalizes to ectopic euchromatin domains (Hildebrand and Biggins, 2016) in a FACT-dependent manner (Deyter and Biggins, 2014). Such proteolysis-mediated regulation of CENP-A levels has also been identified in fission yeast (Yang et al., 2018) and *Drosophila* (Moreno-Moreno et al., 2006). The *Drosophila* CENP-A homologue Cid interacts with Partner of paired (Ppa), an F-box protein, and a component of the Skp, Cullin, F-box containing ubiquitin ligase complex (Moreno-Moreno et al., 2011), involved in controlling Cid stability.

A second putative regulator of CENP-A is the small ubiquitin-like modifier (SUMO) that is structurally related to Ubiquitin. SUMOylation is a dynamic and rapidly reversible posttranslational modification involved in a large number of intracellular pathways including replication, transcription, and DNA repair (Psakhye and Jentsch, 2012). SUMO modifications primarily control protein–protein interactions, whereas a small subset of SUMO modifications, specifically polySUMOylation-mediated polyubiquitination, lead to proteasome-mediated degradation (Flotho and Melchior, 2013). A role for such SUMO-dependent ubiquitination and degradation was uncovered regulating

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CENP-A protein levels. Budding yeast CENP-A^{Cse4} is SUMOylated on its N-terminal tail by SAP and Miz-finger domain-containing protein 1 and 2 (Siz1/2) SUMO E3 ligase (Ohkuni et al., 2016). Siz1 is the founding member of the Siz/PIAS (protein inhibitor of activated STAT) family of SUMO ligases that are involved in SUMOylation of several chromatin substrates including core histones and the replication clamp proliferating cell nuclear antigen (Jentsch and Psakhye, 2013). Cse4 is poly-SUMOylated at lysine 65 in its N-terminal domain (Ohkuni et al., 2018), which recruits the yeast SUMO-targeted ubiquitin ligase (STUbl) Slx5. This in turn mediates the polyubiquitination of poly-SUMOylated Cse4, leading to its subsequent degradation. Similarly, depletion of the human Slx5 homologue ring finger protein 4 (RNF4) was found to mediate SUMOylation-dependent degradation of the CCAN protein CENP-I, resulting in the loss of the CENP-HIK complex from the centromeres (Mukhopadhyay et al., 2010). However, this did not lead to a reduction in centromeric CENP-A or CENP-C levels. Indeed, there is no evidence to date for proteasome-mediated degradation of human CENP-A.

A proteolysis-independent role for SUMOylation in the regulation of CENP-A stability

While some SUMOylation events leads to protein degradation, most regulate protein–protein interactions. SUMO modifications are highly dynamic and are balanced through a large family of SUMO ligases and SUMO proteases (deSUMOylases), the latter cleaving the SUMO protein from the substrate (Nayak and Müller, 2014). In budding yeast, two SUMO proteases are known, ubiquitin-like protease 1 and 2 (Ulp1 and 2), whereas in mammalian cells these have diverged into a large family of Sentrin-specific protease enzymes (SENP1–7). Of these proteins, SENP1–5 are evolutionarily related to Ulp1, while the more divergent SENP6 and SENP7 belong to the Ulp2 branch. A link between the SUMO pathway and the kinetochore was identified almost as early as SUMO itself, which was discovered as SMT3 (suppressor of Mif two 3) along with Ulp2 as high copy suppressors of temperature-sensitive mutations in *MIF2*, the CENP-C homologue in budding yeast (Meluh and Koshland, 1995) and chicken (Fukagawa et al., 2001). Depletion of Ulp2 results in chromosome missegregation including aneuploidy, further indicating that Ulp2 may have a direct role in kinetochore function (Ryu et al., 2016). This notion was borne out by a mass spectrometry–based proteomic screen for Ulp2 substrates that identified the CCAN complex as among the three distinct chromatin-bound protein complexes to be highly enriched for Ulp2 activity, the other two being replication origins and the nucleolus (de Albuquerque et al., 2016). Loss of Ulp2 results in increased SUMOylation of yeast CENP-HIK and -QU by ~20-fold, indicating that these are the specific targets for deSUMOylation by Ulp2. Importantly, expression levels of these CCAN substrates remained unchanged in the Ulp2-null mutant, indicating that polySUMOylation does not lead to ubiquitin-mediated proteolysis. Ulp2 is targeted to the kinetochore by a conserved C-terminal kinetochore targeting motif that binds to CENP-HIK complex, which is further enhanced by SUMO binding via the Ulp2 SUMO interaction motif (SIM; Suhandynata et al., 2019). Loss of Ulp2 results in elevated levels of chromosome missegregation, indicating that aberrant accumulation of polySUMO chains on centromere components results in defective kinetochore function. Strikingly, overexpression of a SUMO mutant that cannot form chains also led to elevated chromosomal missegregation (Suhandynata et al., 2019). These results support the hypothesis not simply that SUMO is detrimental to the kinetochore, but that an optimal level of SUMOylation is needed for kinetochore integrity and function. A speculative role for the many SUMO modifications at the centromere is that they provide a molecular glue that offers robustness to the large and dynamic centromere complex. In this way, centromeres would be akin to promyelocytic (PML) bodies that obtain organelle-like properties (Lin et al., 2006; Shen et al., 2006) through many low-affinity noncovalent SUMO-mediated interactions. However, clearly a tight balance of SUMO levels is needed for maintaining centromere integrity.

Dynamic control ensuring stable transmission of human CENP-A chromatin

The Ulp2 homologue in humans, SENP6, was initially implicated in kinetochore function by deSUMOylating and thereby preventing the polySUMOylation-mediated degradation of CENP-I (Mukhopadhyay et al., 2010). Subsequently, four different studies in the space of a year identified a similar role for SENP6 in regulating the CCAN more broadly. Two of the studies used proteomic approaches to identify SENP6 substrates and/or binding partners. Similar to the yeast results, several CCAN proteins were highly enriched as SENP6 substrates, including direct CENP-A interacting partners CENP-C and -B. Components of the CENP-A loading machinery, including minichromosome instability 18 (A) (MIS18A) and Mis18 binding protein 1 (MIS18BP1), were also found to be substrates of SENP6 (Liebelt et al., 2019). In addition, CENP-H was identified as a SENP6 binding partner, albeit with low confidence (Wagner et al., 2019). Two orthogonal studies identified SENP6 in RNAi screens to identify novel components affecting CENP-A localization to the centromere, either steady-state levels (Fu et al., 2019) or those specifically affecting the localization of ancestral versus newly loaded CENP-A pools, based on SNAP-tag pulse chase imaging (Mitra et al., 2020). Acute degron-mediated depletion of SENP6 resulted in the rapid removal of chromatin-bound CENP-A from the centromere at any stage of the cell cycle (Mitra et al., 2020). This striking observation indicates that the centromeric core is not inherently immobile, as was previously suggested, but that CENP-A stability is continuously ensured by a dynamic SUMO cycle (Fig. 5 A).

Downstream analyses indicated that CENP-A was not a direct substrate of SENP6 (Liebelt et al., 2019; Mitra et al., 2020). Instead, CENP-C and -B were both found to be SENP6 targets, with CENP-C showing loss of localization from the centromere upon hyperSUMOylation. This suggests that the loss in parental CENP-A observed upon SENP6 depletion could be due to the polySUMOylation and resultant mislocalization of the CENP-A binding partners CENP-C and/or -B. However, the effect of SENP6 depletion on CENP-A stability is much greater than observed on depletion of CENP-C or -B alone, indicating that there may be other players that contribute to the SENP6-mediated stabilization of centromeric chromatin. A major outstanding question is how CENP-A, as part of the centromeric nucleosome with strongly multivalent interactions with DNA, can be removed from chromatin upon disassembly of the CCAN. To break intranucleosomal and/or DNA–nucleosome contacts, energy transfer involving ATP-dependent chromatin remodeling complexes is usually required. This could happen naturally as part of the motor activities during replication and transcription. Indeed CENP-C or the CCAN (or both) has been found to act as an anchor maintaining the stability of CENP-A during S phase (Nechemia-Arbely et al., 2019). However, the observation that CENP-A can be dislodged from the centromere at any stage of the cell cycle indicates that additional chromatin remodelers or histone chaperones may regulate the stability of preincorporated CENP-A dynamically throughout the cell cycle, either as a part of chromatin-mediated processes such as transcription or DNA repair or a specific remodeler associated with the centromeric chromatin.

While centromeric chromatin is remarkably stable, the ability to regulate stability and size of the centromere complex is likely important to anticipate fluctuations and damage, ensuring overall protein homeostasis. In somatic cycling cells, maintenance may be more dynamic, as new assembly offers an opportunity to compensate for CENP-A erosion. Conversely, in the face of long-term quiescence such as during meiotic arrest, CENP-A requires exceptional stability (Smoak et al., 2016). Furthermore, the ability to deliberately disassemble centromere components and CENP-A chromatin may be physiologically relevant outside centromeres to prevent erroneous accumulation of centromere proteins in active chromatin. How CENP-A is actually removed from chromatin remains unknown, but we speculate that dynamic control such as though SUMO may offer a means to drive turnover.

Other possible mechanisms that can regulate centromeric chromatin maintenance

The role of chromatin remodelers in CENP-A maintenance

Apart from the disruptive DNA and RNA polymerase motors, other ATP-dependent chromatin remodelers also modify nucleosome structure and positioning to facilitate several essential biological processes such as replication, transcription, DNA repair, and chromosome assembly. The remodeling and spacing factor complex member Rsf1 was the first ATP-dependent motor to be implicated in CENP-A homeostasis. It associates with CENP-A mononucleosomes and appears to be involved in converting newly loaded CENP-A into a stable chromatin form (Perpelescu et al., 2009). Another set of energy consuming enzymes, the small GTPases Cdc42 and Rac and their associated regulator Rac GTPase activating protein 1, have also been implicated in this process (Lagana et al., 2010), enigmatically labeled as "maturation" (Prendergast and Sullivan, 2010), which is still a very poorly understood aspect of CENP-A maintenance that may involve converting CENP-A from a chromatin-bound but immature form to a full-blown octameric nucleosome.

The sucrose nonfermentable 2 (SNF2) superfamily helicase in fission yeast Fun30 (Fft3) was found to suppress histone turnover in heterochromatic regions, including pericentric heterochromatin, and help in the epigenetic inheritance of the heterochromatic state (Taneja et al., 2017). Similarly, the human homologue, SWI/SNF related, matrix-associated actin-dependent regulator of chromatin 1 (SMARCAD1), was found to localize to pericentric heterochromatin during centromeric DNA replication (Rowbotham et al., 2011). Absence of SMARCAD1 leads to reduced H3K9Me3 in the pericentromeric chromatin as well as increased instances of DNA bridges and lagging chromosomes, indicating that SMARCAD1-mediated maintenance of pericentric heterochromatin silencing is important for proper chromosome segregation. SMARCAD1 was also found to be a candidate for the maintenance of preincorporated CENP-A in a genetic screen (Mitra et al., 2020). It is tempting to speculate that SMARCAD1 performs a function similar to that of Fft3 by suppressing the turnover of parental CENP-A nucleosomes and facilitating their recycling at the centromere during DNA replication (Fig. 5 D).

The role of centromeric DNA and DNA repair factors in CENP-A maintenance

A distinguishing feature of the centromere is the underlying DNA that is primarily composed of higher-order arrays of 171-bp α-satellite repeats. This repetitive DNA is prone to form secondary structures including hairpins (Zhu et al., 1996) that are known to cause stalling and subsequent collapse of replication forks, often resulting in the formation of double-strand breaks (DSBs; Branzei and Foiani, 2010). Genome-wide mapping of DSBs by next-generation sequencing revealed that α-satellite repeats are enriched in DSBs (Crosetto et al., 2013). Further, proteome analysis of replicating centromeres using bacterial artificial chromosomes containing human α-satellite DNA revealed an enrichment of DNA repair factors, including members of the mismatch repair complex, MutS homologue 2–6 (MSH2-6), and members of the DSB repair Mre11-Rad50-Nbs1 (MRN) complex (Aze et al., 2016). Further, it was found that MSH2–6 was essential for efficient replication of the α-satellite DNA. MSH2–3 has been shown previously to bind to secondary structures such as DNA hairpins (Owen et al., 2005). Combined, these studies indicate that centromeric DNA is prone to DNA damage during replication and requires the active participation of DNA repair proteins to complete replication. DNA repair also involves remodeling enzymes to render chromatin accessible for the loading of effector proteins to repair damage. Interestingly, SMARCAD1 was found to evict nucleosomes around a mismatched base pair site in coordination with MSH2 (Terui et al., 2018). Both SMARCAD1 and PMS2 (postmeiotic segregation increased 2), which performs the role of endonuclease in the mismatch repair complex pathway, were identified as candidates for parental CENP-A maintenance in a genetic screen (Mitra et al., 2020; Fig. 5 D). These findings may point at an, as of yet, unexplored aspect of CENP-A maintenance.

Conclusions and future perspectives

CENP-A nucleosomes are transmitted through multiple cell cycles despite nuclear processes that potentially disrupts centromeric chromatin. The high stability of CENP-A is mediated in part by the structural features encoded within CENP-A, conferring unique biophysical properties. Further, the

CENP-A–associated CCAN proteins likely play a dominant role in stabilizing CENP-A nucleosomes throughout the cell cycle. However, the CCAN structure is dynamic, with new deposition of individual CCAN components occurring at different stages of the cell cycle (Hellwig et al., 2011; Hemmerich et al., 2008). Posttranslational modifications of CCAN proteins also lead to changes in the CCAN architecture, as observed by SUMOylation of multiple CCAN subunits (Mitra et al., 2020; Liebelt et al., 2019) and phosphorylation of CENP-C (Watanabe et al., 2019). In our view, future directions will involve determining how the compositional and structural changes in the CCAN impact the stability of CENP-A nucleosomes throughout the cell cycle. Another important advance will be to develop methodologies to directly track the local dynamics of CENP-A nucleosomes at the replication fork. Recently, a CRISPR-targeting pulse biotinylation system was developed to track canonical parental nucleosome segregation at single loci (Escobar et al., 2019). An analogous approach for CENP-A involves the challenge of designing guide RNAs for repetitive α-satellite DNA that constitutes the native centromeric region. In this instance, neocentromeres, atypical centromeres often formed spontaneously on unique sequences, will serve as a useful resource to study local parental CENP-A dynamics. Finally, it is notable that to date, with the exception of RNA polymerase, very few energy-consuming enzymes are implicated in centromere assembly and dynamics. An important question going forward is to understand the role of ATP-dependent chromatin remodelers at the centromere. This will be key to understanding the exact nature of the active process that enables control of the choice between turnover or stable transmission of CENP-A nucleosomes across the cell cycle and inheritance of centromere identity.

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