Chromatin assembly: Journey to the CENter of the chromosome

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All eukaryotic genomes are packaged into basic units of DNA wrapped around histone proteins called nucleosomes. The ability of histones to specify a variety of epigenetic states at defined chromatin domains is essential for cell survival. The most distinctive type of chromatin is found at centromeres, which are marked by the centromere-specific histone H3 variant CENP-A. Many of the factors that regulate CENP-A chromatin have been identified; however, our understanding of the mechanisms of centromeric nucleosome assembly, maintenance, and reorganization remains limited. This review discusses recent insights into these processes and draws parallels between centromeric and noncentromeric chromatin assembly mechanisms.

Introduction

At its simplest level, the organization of chromatin in the genome consists of individual DNA molecules wrapped around histone proteins (Kornberg, 1974; Olins and Olins, 1974). Together they form the nucleosome particle, which contains one $(H3\text{-}H4)_2$ tetramer and two H2A-H2B dimers (Luger et al., 1997). In recent years, it has become clear that the presence of histone modifications and the deployment of histone variants underlie specific genomic events, yet we are only beginning to understand how these defined chromatin domains are nucleated and how defects in their organization affect genome function.

Although we can now determine the composition of chromatin genome-wide, our ability to predict the functional output of defined regions from their epigenetic profile remains limited. One striking exception to this is centromeric chromatin, in which the presence of the histone H3 variant CENP-A (Earnshaw and Rothfield, 1985; also known as CenH3 [Talbert and Henikoff, 2013]) is both necessary and sufficient to confer centromere activity to any genomic region, whether or not it contains centromeric DNA sequences (Saffery et al., 2000; Heun et al., 2006; Mendiburo et al., 2011). This remarkable connection between a unique type of chromatin and the highly specialized function it encodes has fascinated chromosome biologists for more than three decades.

Many aspects of the structure and functions of centromeric chromatin have been elucidated, but much is still unknown, particularly concerning how centromeric nucleosomes are reorganized during centromere establishment and maintenance. In general terms, the process that leads to nucleosome formation consists of steps that are shared between all different types of histone H3-containing nucleosomes. Nucleosomes are assembled from H3-H4 dimeric precursors (Tagami et al., 2004), which form $(H3\text{-}H4)_2$ tetramers. The deposition of these tetramers onto DNA is followed by the sequential addition of two H2A-H2B dimers (Nakagawa et al., 2001). Because histones are unable to self-assemble into nucleosomes under physiological conditions and tend to interact with DNA nonspecifically (Wilhelm et al., 1978), histone chaperones (or assembly factors) are required to assist in their assembly and disassembly by regulating the interactions between histones and DNA (Haushalter and Kadonaga, 2003). Assembly factors in complex with histone dimers are then recruited to sites of chromatin assembly by chromatin-associated proteins, where they are deposited. After the formation of new nucleosomes, chromatin organization is restored by chromatin remodeling complexes, which promote nucleosome spacing. An overview of this process is shown in Fig. 1.

In this review, we discuss our current understanding of the mechanisms of centromere chromatin assembly and maintenance. We focus on the complex centromeres of metazoans, drawing functional comparisons with the corresponding mechanisms of deposition and maintenance of canonical histone H3 (H3.1; broadly associated with the entire genome) and the replacement variant H3.3 (associated with transcriptionally active regions and repetitive DNA; Loyola and Almouzni, 2007). We also examine recent advances that have shed light on the dynamic chromatin transitions involving transcription and histone acetylation that occur during CENP-A deposition.

CENP-A nucleosomes confer centromere activity

During mitosis and meiosis, nucleosomes containing CENP-A demarcate the genomic location where the kinetochore assembles (McKinley and Cheeseman, 2016). A defective centromere/kinetochore complex results in the incorrect segregation of chromosomes, which is linked to developmental abnormalities, miscarriages, and cancer (Ehrlich et al., 2006; Amato et al., 2009).

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Abbreviations used in this paper: ASF1, anti-silencing factor 1; CAF1, chromatin assembly factor 1; CATD, CENP-A targeting domain; FACT, facilitates chromatin transcription; HFD, histone fold domain; HIRA, histone regulatory homologue A; RSF, remodeling and spacing factor.

The Rockefeller University Press $30.00
J. Cell Biol. Vol. 214 No. 1 13–24
www.jcb.org/cgi/doi/10.1083/jcb.201605005

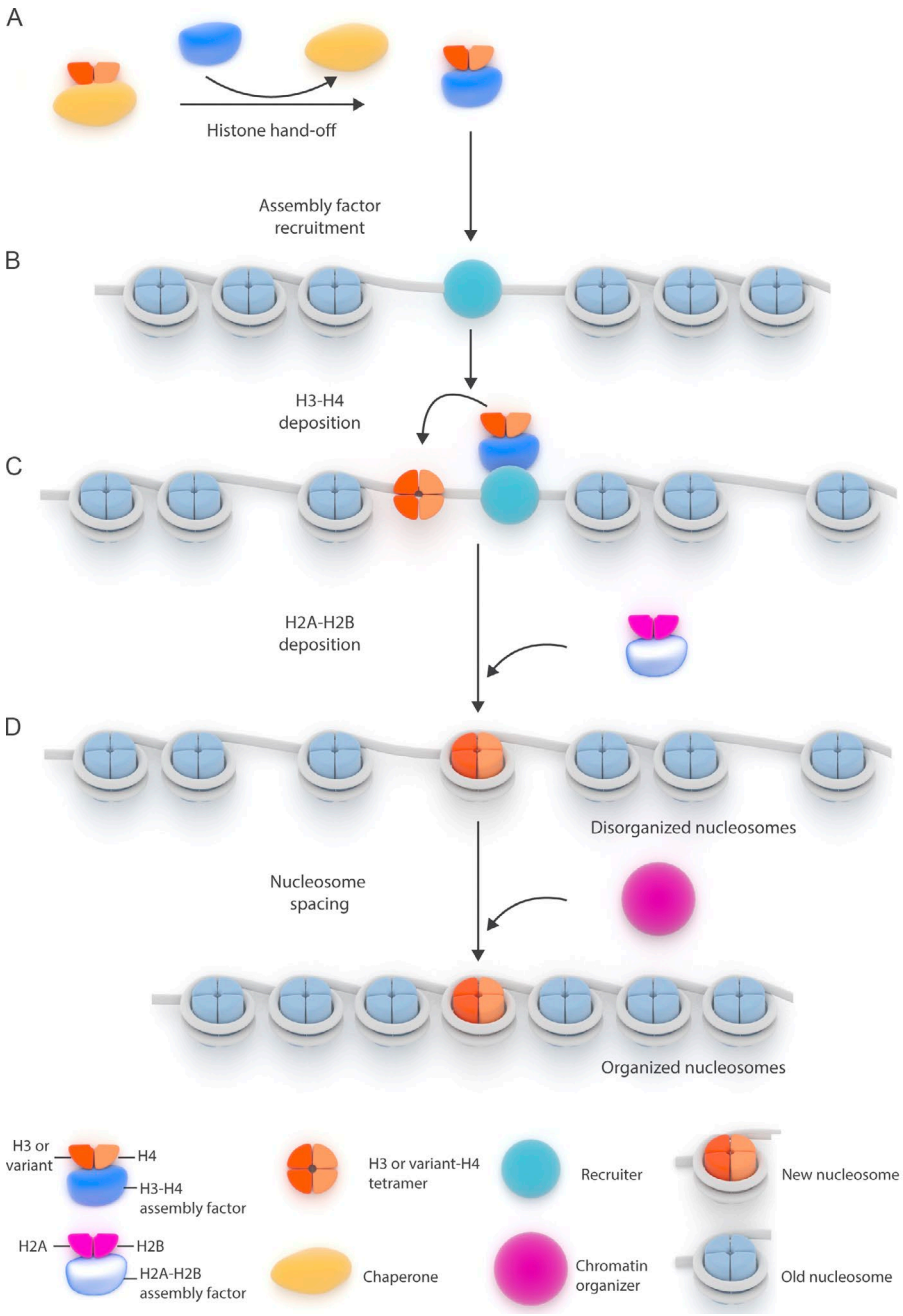

Figure 1. **Overview of the stepwise assembly of histone proteins onto chromatin.** (A) Nucleosomes form from H3-H4 dimer precursors, which associate with histone chaperones that prevent nonspecific histone–DNA interactions. These chaperones hand off histone dimers to assembly factors, which are specific to the histone they recognize. (B) The H3-H4 assembly factor is recruited to target chromatin regions by DNA-bound recruiter proteins, allowing the deposition of $(H3\text{-}H4)_2$ tetramers. (C) Upon the incorporation of H2A-H2B dimers, the new nucleosome forms. (D) The spacing of new and old particles that might have been altered during new histone deposition (disorganized nucleosomes) is restored by the chromatin remodeling activity of chromatin organizers (organized nucleosomes).

In plants and animals, centromeric chromatin is not uniformly composed of CENP-A nucleosomes, but instead contains interspersed domains of CENP-A nucleosomes alternating with stretches of H3 nucleosomes (Blower et al., 2002; Greaves et al., 2007; Yan and Jiang, 2007). The functional significance of this arrangement is unknown, but models of how it may contribute to the three-dimensional organization of centromeric chromatin have been proposed (Blower et al., 2002; Ribeiro et al., 2010).

As a histone that marks the centromere, CENP-A must confer unique properties to the nucleosomes that contain it, enabling it to mediate the specific recruitment of centromere and kinetochore proteins. Furthermore, the distinct properties of CENP-A nucleosomes are likely to be critical for its exclusively centromeric deposition and to prevent aberrant assembly of CENP-A at noncentromeric locations. Thus, a comparative understanding of the biophysical properties of CENP-A nucleosomes is of critical importance to understand accurate genome inheritance.

Like all histone proteins, the C terminus of CENP-A contains a globular histone fold domain (HFD), which consists of three α-helices linked by two loops (Arents et al., 1991). A region encompassing loop 1 and the α2-helix of CENP-A, known as the CENP-A targeting domain (CATD), is required for the binding of CENP-A to centromeres and sufficient for conferring centromere localization to a histone H3 chimera that contains it (Vermaak et al., 2002; Black et al., 2004, 2007b). A comprehensive illustration showing the differences and similarities between histone H3 and CENP-A can be found in a recent review (McKinley and Cheeseman, 2016).

The CATD mediates the recognition of CENP-A by its specific assembly factor, called HJURP (Holliday junction recognition protein; Dunleavy et al., 2009; Foltz et al., 2009) in

tetrapods and Scm3 (suppressor of chromosome missegregation protein 3; Camahort et al., 2007; Mizuguchi et al., 2007; Stoler et al., 2007; Pidoux et al., 2009; Sanchez-Pulido et al., 2009) in fungi. Flies use an evolutionarily distinct CENP-A assembly factor called CAL1 (chromosome alignment defect 1; Goshima et al., 2007; Erhardt et al., 2008; Phansalkar et al., 2012; Chen et al., 2014), whereas nematodes, plants, fish, and other arthropods lack clear homologues of any of these CENP-A assembly factors (Sanchez-Pulido et al., 2009). For *Drosophila melanogaster* CENP-A, loop 1 has been shown to specify recognition by CAL1 (Rosin and Mellone, 2016), whereas a CATD remains poorly defined (Moreno-Moreno et al., 2011).

CENP-A harbors several physical features that distinguish it from histone H3. In humans, the HFD of CENP-A is more than 60% identical to that of canonical H3, whereas the N-terminal tail bears no sequence similarity with H3 (Sullivan et al., 1994). Additionally, CENP-A nucleosomes harbor looser DNA termini (Hasson et al., 2013), form particles that appear smaller by atomic force microscopy (Dalal et al., 2007; Miell et al., 2013), and display higher rigidity compared with their canonical H3 counterparts (Black et al., 2007a).

In recent years, conflicting data have been reported regarding the precise composition and handedness of CENP-A nucleosomes. Studies in yeast, flies, and humans supported the existence of a nonoctameric particle containing one CENP-A-H4 and one H2A-H2B dimer (called a hemisome) with DNA wrapped right-handedly (Henikoff and Furuyama, 2012; Bui et al., 2013). However, a growing number of studies, in vitro and in vivo, have challenged that model, strongly favoring an octameric CENP-A particle akin to the left-handed histone H3 in humans and flies (Black and Cleveland, 2011; De Rop et al., 2012; Dunleavy et al., 2013). The initial proposal that human CENP-A nucleosomes experience cell cycle–dependent size fluctuations between hemisome and octamer configurations (Bui et al., 2012) has also been challenged by data demonstrating constant histone composition throughout the cell cycle (Hasson et al., 2013; Padeganeh et al., 2013). Interestingly, a recent study showed that the human centromere contains positively supercoiled DNA. However, this overwinding of centromeric DNA appears to be mediated by topoisomerase I, rather than by the presence of right-handed CENP-A particles (Aze et al., 2016). A hemisome structure, on the other hand, explains both the positive supercoiling and the cleavage mapping of the CENP-A nucleosome of budding yeast centromeres in vivo (Furuyama et al., 2006; Henikoff et al., 2014).

Additional insights into the properties of the human CENP-A particle have emerged from analysis of the crystal structure of the CENP-A octamer. The HFD of CENP-A contains a shorter αN-helix compared with histone H3. This might be important for the conformation of the DNA at the entrance and exit of CENP-A nucleosomes and might have implications for allowing DNA-binding proteins, such as CENP-B, to associate with CENP-A nucleosomes (Earnshaw and Rothfield, 1985). Furthermore, loop 1 of human CENP-A contains two extra amino acids compared with H3, which are critical for the stability of the CENP-A nucleosome and might provide a binding site for regulatory factors (Tachiwana et al., 2011).

An additional CENP-A–specific feature is its unstructured C-terminal tail, which, in *Xenopus laevis*, is specifically recognized by the inner kinetochore protein CENP-C (Saitoh et al., 1992; Guse et al., 2011). The C-terminal tail of CENP-A is necessary for maintaining centromere identity, as H3 chimeras containing both the CATD and the C-terminal tail of CENP-A can sustain long-term centromere function (Fachinetti et al., 2013). However, the C-terminal tail is not conserved across species (for example, it is 6 aa long in humans, 1 aa in *Schizosaccharomyces pombe*, and 3 aa in *Drosophila*). Whether or not the C-terminal tail of CENP-A is also recognized by CENP-C in these species remains to be determined. Collectively, the distinct structural and biophysical properties of CENP-A nucleosomes epigenetically specify the unique role of centromeric chromatin, distinguishing it from any other region of the genome.

Parental CENP-A nucleosome retention and redistribution in S phase

The repackaging of daughter DNA strands into chromatin during replication occurs rapidly (Gasser et al., 1996). Parental (i.e., "old") histone $(H3.1\text{-}H4)_2$ tetramers are transferred behind the replication fork in a random fashion to either DNA duplex (Sogo et al., 1986; Jackson, 1988), and nascent $(H3.1\text{-}H4)_2$ tetramers are deposited in the gaps left during this process (Jackson and Chalkley, 1985). Thus, after DNA replication, canonical nucleosomes consist of either entirely new or entirely parental tetramers (Yamasu and Senshu, 1990). Although the factors that are responsible for placing the parental H3.1-H4 histones back into chromatin during replication are not fully known, chromatin-associated anti-silencing factor 1 (ASF1) and the facilitates chromatin transcription (FACT) complex have been found to associate with parental histones (Groth et al., 2007; Foltman et al., 2013), and thus might contribute to their retention (Fig. 2 A).

For some genomic loci, parental histones carry specific epigenetic information, the retention of which during replication is essential for specifying various chromatin identities. This is particularly important for CENP-A chromatin, the loss of which results in the failure to demarcate the point of attachment by spindle microtubules in mitosis and meiosis, leading to chromosome loss (McKinley and Cheeseman, 2016).

A photo-bleaching, single-molecule counting method has shown CENP-A to be present in two copies in each nucleosome throughout the cell cycle, suggesting that CENP-A is retained as a tetramer, without dimer intermediates through DNA replication (Padeganeh et al., 2013; Fig. 2 B). The gaps left by the redistribution of parental CENP-A nucleosomes are temporarily occupied by histone H3.1 and H3.3, which are deposited during S phase as possible gap-fillers (Dunleavy et al., 2011).

As with the mechanisms of retention of parental $(H3.1\text{-}H4)_2$ tetramers, very little is known about the mechanism of CENP-A maintenance at the centromere. The retention of parental CENP-A across cell division (Jansen et al., 2007; Mellone et al., 2011) and the paucity of CENP-A exchange detected by FRAP experiments (Hemmerich et al., 2008) suggest that CENP-A is actively retained at centromeres during DNA replication. Indeed, pulse-chase experiments have demonstrated that parental CENP-A nucleosomes outlast their histone H3.1 and H3.3 counterparts, indicating that noncentromeric chromatin experiences higher turnover. Intriguingly, the high stability of CENP-A through multiple cell cycles is encoded within the CATD, even though it does not require HJURP (Bodor et al., 2013). The physical properties conferred by the CATD, or an as-yet-unidentified factor interacting with it, could confer high stability and prevent CENP-A nucleosomes from dissociating during DNA replication (Fig. 2 B).

Other properties of centromeric chromatin, or of chromatin in its vicinity, might contribute to the retention and stability

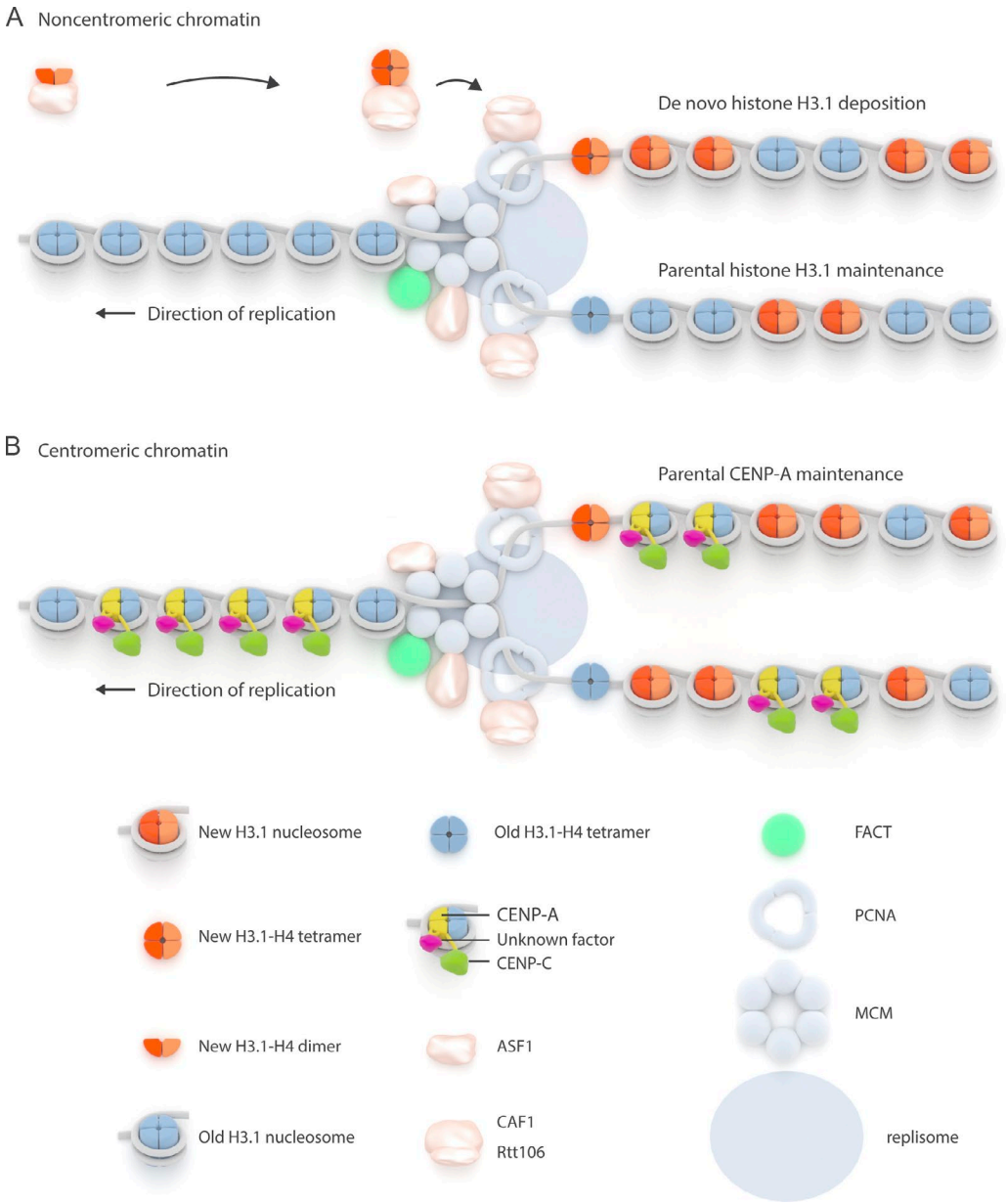

Figure 2. **Histone deposition and retention during DNA replication.** (A) De novo histone H3.1-H4 deposition is initiated when new H3.1-H4 dimer precursors interact with the chaperone ASF1. ASF1 then hands them to the assembly factor CAF1 in complex with Rtt106. The $(H3.1\text{-}H4)_2$ tetramer then forms and is deposited onto DNA via an interaction between CAF1 and the recruiter protein, proliferating cell nuclear antigen (PCNA). At the replication fork, FACT is associated with the minichromosome maintenance (MCM) complex to promote chromatin disassembly. Parental $(H3.1\text{-}H4)_2$ tetramers are transferred behind the replication fork in a random fashion, possibly aided by FACT and ASF1. (B) During S phase, parental $(CENP\text{-}A\text{-}H4)_2$ tetramers are depicted as being retained as intact molecules, consistent with the observed retention of preexisting CENP-A at replicated centromeres. The stability of CENP-A nucleosomes through multiple cell cycles is encoded within the CATD, through either the specific physical characteristics it confers or its interaction with an unknown factor. CENP-C binds to the C-terminal tail of CENP-A, further stabilizing the CENP-A nucleosome, which might be important for its retention through S phase.

of CENP-A through DNA replication. Condensin II has multiple roles in the regulation of interphase chromatin and was found to stabilize *Xenopus* CENP-A nucleosomes (Bernad et al., 2011). Furthermore, interference with normal condensin II degradation in *Drosophila* cells results in the dispersion of CENP-A foci (Buster et al., 2013). Whether the role of condensin II in CENP-A stability is specific to S phase remains to be established, but clearly, altering chromatin condensation affects centromeric chromatin integrity.

CENP-A retention may be enhanced by its interactions with binding partners. CENP-C is required for the stability of CENP-A nucleosomes (Falk et al., 2015, 2016) and is thus a primary candidate for a role in stabilizing CENP-A nucleosomes during DNA replication (Fig. 2 B). Furthermore, chromatin assembly factor 1 (CAF1) subunits RbAp46/48 in humans and p55 in *Drosophila* mediate chromatin assembly during DNA replication (Smith and Stillman, 1989; Fig. 2 A) and interact with CENP-A (Foltz et al., 2006; Furuyama et al., 2006; Dunleavy et al., 2009; Shuaib et al., 2010), suggesting that CAF1 might play a role in CENP-A maintenance during S phase. So far, this interaction has been detected only in soluble (i.e., not yet chromatin incorporated) or cytoplasmic CENP-A;

therefore, it remains to be established whether this complex puts CENP-A back onto DNA during DNA replication, as observed for histone H3.1. Thus, a combination of the regulation of chromatin architecture (e.g., condensin II) and the presence of interacting partners (e.g., CENP-C, RbAp46/48, and p55) could ensure that CENP-A is not lost from parental nucleosomes during DNA replication.

Nascent CENP-A deposition is uncoupled from replication

Histone H3.1 is deposited in a DNA replication–dependent manner (Wu and Bonner, 1981; Jackson, 1988), whereas the variant H3.3 is deposited onto chromatin throughout the cell cycle, independently of DNA synthesis (Hake and Allis, 2006; Hake et al., 2006).

Controlling when newly synthesized CENP-A is deposited has long been proposed to be a means to distinguish centromeric from bulk chromatin composed of histone H3 nucleosomes (Ahmad and Henikoff, 2002). CENP-A is consistently incorporated into chromatin outside of S phase in most organisms (McKinley and Cheeseman, 2016), with the exception of budding yeast (Pearson et al., 2004). The timing of CENP-A deposition differs among species; CENP-A is deposited between mitosis and G1 in *Drosophila* (Schuh et al., 2007; Mellone et al., 2011; Dunleavy et al., 2012; Lidsky et al., 2013), between G2 and prophase in *Dictyostelium discoideum* (Dubin et al., 2010), and in G2 in *Arabidopsis thaliana* (Lermontova et al., 2006). Fission yeast can deposit CENP-A during both S phase and G2 (Takahashi et al., 2005), whereas in human cells and *Xenopus* egg extracts, CENP-A is deposited from late telophase through G1 (Jansen et al., 2007; Bernad et al., 2011).

The cell cycle timing of CENP-A deposition results from an interplay between cell cycle regulators and the CENP-A loading machinery. CENP-A deposition is antagonized by the activity of cyclin dependent kinase 1 (Cdk1) in human and *Drosophila* cells (Mellone et al., 2011; Silva et al., 2012; Yu et al., 2015). After its specific loading window, CENP-A is constantly maintained at the centromere (Bodor et al., 2013). A GTPase-activating protein, male germ cell RacGap, is required for this maintenance (Lagana et al., 2010).

Prenucleosomal CENP-A is in complex with histone H4 (Foltz et al., 2009), and in human cells, newly synthesized CENP-A and H4 are detected at centromeres in G1, suggesting that their deposition occurs in the form of an intact nascent complex (Bodor et al., 2013). HJURP and Scm3 bind CENP-A-H4 dimers, preventing the formation of $(\text{CENP-A-H4})_2$ tetramers (Cho and Harrison, 2011; Feng et al., 2011; Hu et al., 2011). Therefore, $(\text{CENP-A-H4})_2$ tetramers are thought to form via the self-dimerization of the HJURP/CENP-A/H4 complex, followed by their deposition (Zasadzińska et al., 2013). However, how the final octameric structure of CENP-A nucleosomes forms remains elusive. Fig. 3 outlines two possible models. In one model, parental $(\text{H3-H4})_2$ tetramers are replaced with new $(\text{CENP-A-H4})_2$ tetramers within intact nucleosomes that contain old H2A-H2B dimers. In the other, preexisting H3-containing centromeric nucleosomes are disassembled and reassembled by incorporating new $(\text{CENP-A-H4})_2$ tetramers and a mix of old and new H2A-H2B dimers. The fact that nascent H2B at the centromeres incorporates at a rate that is indistinguishable from that of the rest of the genome (Bodor et al., 2013) is consistent with either model. Regardless of the specific mechanism of nucleosome assembly, these new CENP-A particles must replenish the halved CENP-A complement of replicated sister chromosomes (Jansen et al., 2007; Mellone et al., 2011).

Although two recent imaging-based quantitative studies seem to differ on whether sister centromeres contain consistently similar or different amounts of CENP-A in metaphase (Bodor et al., 2014; Ross et al., 2016), which has implications for our understanding of the mechanisms of CENP-A redistribution during S phase, it is clear that, in G1, new CENP-A is deposited within preexisting CENP-A domains, whereas interspersed histone H3 stretches do not experience CENP-A exchange (Ross et al., 2016).

CENP-A hand-off and recruitment

Before being assembled into nucleosomes, histones need to be delivered to the assembly factor that will deposit them at their designated chromatin regions (Fig. 1). Nascent H3.1-H4 dimers are associated with ASF1 and, after nuclear import, are handed off to CAF1 (Tyler et al., 2001; Fig. 2 A). Likewise, newly synthesized H3.3-H4 dimers are handed off by ASF1 to the H3.3-specific chaperones histone regulatory homologue A (HIRA; Daniel Ricketts et al., 2015) or death domain–associated protein (DAXX; Elsässer et al., 2012) for their recruitment at transcribed regions or at the telomeres and heterochromatin, respectively (Drané et al., 2010; Goldberg et al., 2010; Lewis et al., 2010). However, the factors that hand off CENP-A to its specific chaperones, HJURP/Scm3 and CAL1, are unknown, and human CENP-A does not appear to associate with ASF1 (Dunleavy et al., 2009). CAF1 is recruited to DNA through an interaction with proliferating cell nuclear antigen close to the replication fork (Shibahara and Stillman, 1999) and mediates the deposition of nascent $(\text{H3.1-H4})_2$ tetramers throughout the genome (Fig. 2 A).

Several factors recruit the H3.3 chaperone HIRA to chromatin, including the GAGA factor, which targets HIRA to GAGA factor binding sites in *Drosophila* (Nakayama et al., 2007). Conversely, for the H3.3 chaperone, DAXX, the only known recruiting complex is the chromatin remodeler α-thalassemia/mental retardation syndrome protein (ATRX), which targets DAXX to heterochromatic regions in mouse embryonic stem cells (Lewis et al., 2010; Voon and Wong, 2016). Thus, the assembly factors for histone H3.1 and H3.3 are recruited to specific chromatin domains through their interaction with distinct partners.

Unlike global histone H3 deposition, targeting of CENP-A by its specific assembly factors features both spatial and temporal control, which strictly govern its deposition at centromeres to prevent the formation of spurious kinetochore attachments (Valente et al., 2012). In human cells, HJURP is recruited to centromeric chromatin by the Mis18 complex, composed of hMis18α, hMis18β, and Mis18BP1, during mitotic exit (Fujita et al., 2007; Barnhart et al., 2011). The Mis18 complex is recruited to centromeres via an interaction with centromere-bound CENP-C (Moree et al., 2011; Dambacher et al., 2012). The formation of an hMis18α-hMis18β heterotetramer (and a Mis18 homotetramer in *S. pombe*) is required for centromere binding of the Mis18 complex. Upon HJURP binding, the heterotetramer is disrupted, causing the Mis18 complex to dissociate from the centromere, thereby limiting CENP-A loading to a one-time event per cell cycle (Nardi et al., 2016; Subramanian et al., 2016). Additional regulation occurs through Cdk1/Cdk2 activity, which inhibits the assembly of the Mis18 complex until anaphase onset, and phosphorylation of Mis18BP1 by Polo-like

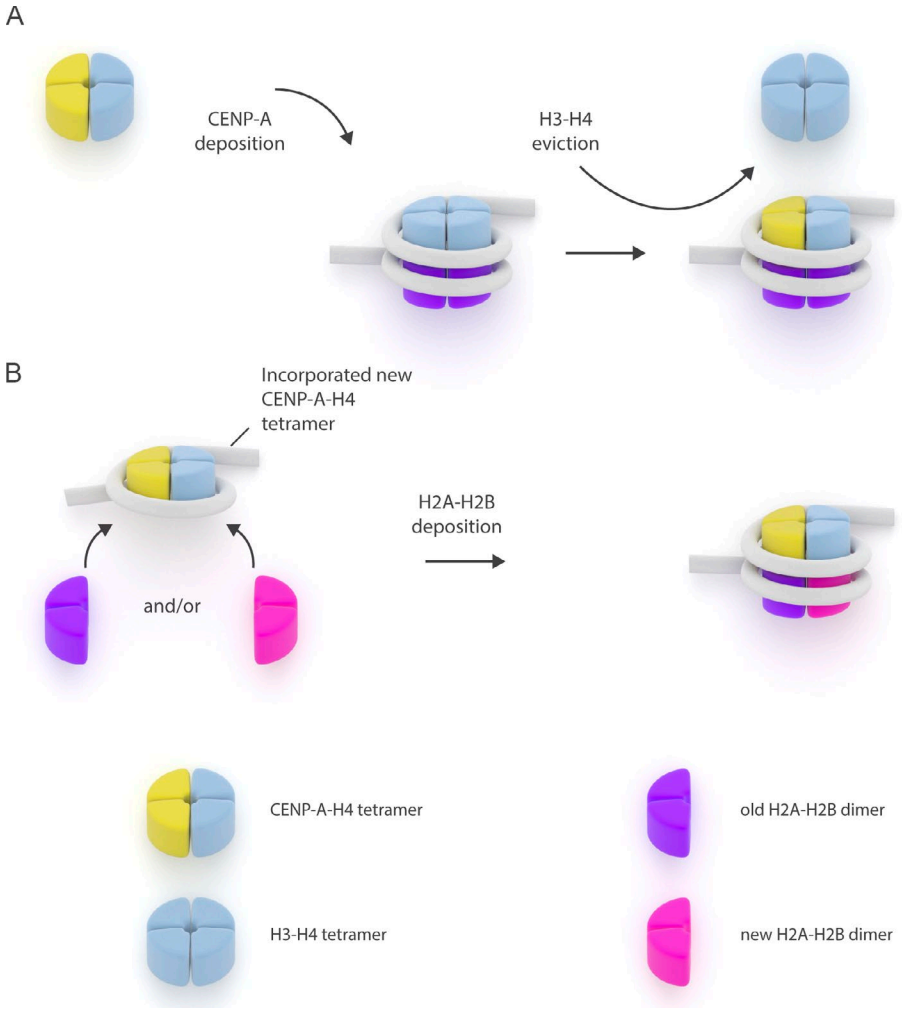

Figure 3. **Possible mechanisms for the formation of CENP-A nucleosomes.** (A) CENP-A is deposited in its tetrameric $(CENP\text{-}A\text{-}H4)_2$ form onto DNA. The CENP-A octamer might form via the eviction of the placeholder $(H3.1/H3.3\text{-}H4)_2$ tetramers, within intact nucleosomes containing old H2A-H2B dimers. (B) Alternatively, preexisting H3.1/H3.3-containing nucleosomes could be entirely disassembled from DNA, with new $(CENP\text{-}A\text{-}H4)_2$ tetramers deposited, followed by the assembly of H2A-H2B dimers from a pool of old and new ones.

kinase 1 promotes its association with CENP-C, resulting in centromere recruitment (McKinley and Cheeseman, 2014).

The recruitment machinery for the CENP-A/H4-chaperone complex is conserved in fission yeast and *Caenorhabditis elegans* (Hayashi et al., 2004; Maddox et al., 2007). However, the Mis18 complex is absent in *Drosophila*, where the centromeric localization of the CENP-A assembly factor CAL1 depends solely on CENP-C (Chen et al., 2014).

Chromatin reorganization during CENP-A replacement

The recruitment of histone H3 and its variants at the site designated for assembly must be coupled with the reorganization of local chromatin to (a) displace unwanted histones or nucleosomes; (b) make room for new nucleosomes; and/or (c) respace nucleosomes that have shifted during new nucleosome assembly (Fig. 1 C). How chromatin is remodeled during CENP-A deposition is poorly understood. The chaperones HJURP, Scm3, and CAL1 have been shown in vitro to harbor selective affinity for CENP-A over histone H3 and to mediate CENP-A nucleosome formation on naked DNA plasmids (Barnhart et al., 2011; Cho and Harrison, 2011; Chen et al., 2014). However, it is unclear whether these assembly factors can reorganize preexisting H3.1/H3.3-containing nucleosomes to make room for CENP-A. DNA–histone interactions need to be altered for this purpose, and several mechanisms have been identified that can perform these changes, such as the deployment of ATP-dependent histone remodelers and histone chaperones and the passage of DNA replication and transcription machineries (Gruss et al., 1993; De Koning et al., 2007; Liu et al., 2010; Ransom et al., 2010; Kharchenko et al., 2011).

For histone H3.3 deposition during *Drosophila* embryogenesis, HIRA relies on the ATP-dependent remodeler protein chromodomain helicase DNA binding protein 1 (CHD1) for global chromatin reorganization (Konev et al., 2007), whereas in murine embryonic stem cells, DAXX cooperates with the chromatin-remodeling factor ATRX to incorporate H3.3-H4 dimers at repetitive chromatin regions (Lewis et al., 2010). Thus, the cooperation between H3.3 assembly factors and ATP remodelers suggests that nucleosome reorganization is necessary for histone replacement. Consistent with this idea, the remodeler CHD1 has been shown to localize to the centromere and to be required for the localization of CENP-A in fission yeast and chicken cells (Walfridsson et al., 2005; Okada et al., 2009), suggesting that this ATPase/helicase contributes to CENP-A chromatin formation (Fig. 4). CHD1 is apparently not required for proper CENP-A localization in flies (Podhraski et al., 2010), suggesting that other chromatin remodelers might be used in different species for this purpose.

Another chromatin remodeler, the remodeling and spacing factor (RSF) complex, is also implicated in centromere chromatin function, specifically in the process of CENP-A nucleosome

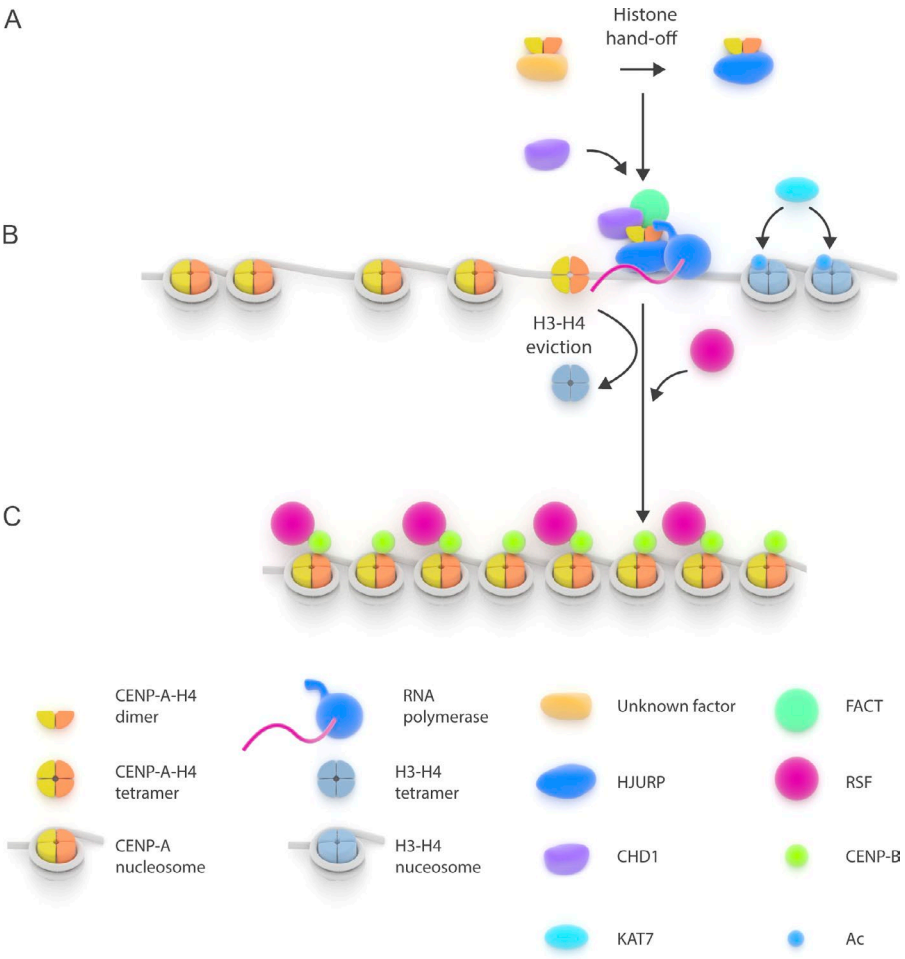

Figure 4. **Chromatin reorganization during CENP-A assembly in humans.** (A) Newly synthesized CENP-A-H4 dimers in complex with an as-yet-unidentified chaperone are handed off to the assembly factor HJURP, which is recruited to the centromere by the Mis18 complex–CENP-C pathway (not depicted). (B) After the recruitment of CHD1, FACT, and RNA polymerase II, the placeholder (H3.1/H3.3-H4)$_2$ tetramers (or entire nucleosomes) are disassembled and (CENP-A-H4)$_2$ tetramers are deposited onto DNA. Mis18 transiently recruits the lysine acetyltransferase KAT7 to centromeres, which acetylates H3-containing nucleosomes and facilitates RSF binding. (C) After their formation, new CENP-A nucleosomesare spaced by the RSF complex, which promotes CENP-B binding.

incorporation and in nucleosome spacing. In human cells, CENP-A nucleosomes display specific positioning, binding to α-satellite DNA, such that the CENP-B box is adjacent to the proximal edge of the nucleosome (Hasson et al., 2013). This positioning enhances the binding of CENP-B, which stabilizes the CENP-A nucleosomes (Fujita et al., 2015). How this positioning is accomplished is unknown, but it could involve the ATP-dependent spacing activity of RSF, given its ability to space CENP-A nucleosomes in vitro and its interaction with CENP-B (Perpelescu et al., 2009; Fig. 4 C).

The two RSF subunits, RSF1 and SNF2h, associate with human CENP-A (Obuse et al., 2004; Izuta et al., 2006; Perpelescu et al., 2009), are enriched at centromeres in interphase, and are required for CENP-A chromatin incorporation in vivo and for assembly of CENP-A nucleosomes in vitro (Perpelescu et al., 2009). RSF was recently shown to be recruited to centromeres by a novel mechanism involving the transient acetylation of centromeric chromatin by the lysine acetyl transferase KAT7, which is recruited by Mis18BP1 during G1. Recruitment of KAT7 also stimulates transcription (Ohzeki et al., 2016; Fig. 4 B), a process that has been gaining attention as an additional mechanism by which chromatin can be reorganized during centromeric chromatin assembly.

Transcription-mediated chromatin reorganization

During transcription, the passage of RNA polymerases along the DNA increases histone exchange (Jackson, 1990). Consistent with this model is that the association between HIRA and transcriptional elongation factors promotes the replacement of H3.1 with H3.3 (Nakayama et al., 2007; Ray-Gallet et al., 2011; Sarai et al., 2013), suggesting that HIRA-mediated H3.3 deposition might replenish histone H3-H4 complexes that were lost during transcription, thereby maintaining chromatin integrity. The role of transcription in promoting centromere integrity is emerging as an important and potentially conserved mechanism of centromere regulation (Fig. 4; Chan and Wong, 2012; Gent and Dawe, 2012; Hall et al., 2012; Rošić and Erhardt, 2016).

Intriguingly, the active form of RNA polymerase II has been found to be enriched at the centromeres of several organisms (Chan et al., 2012; Rošić et al., 2014; Catania et al., 2015) and, in flies, it was shown to physically interact with CAL1, suggesting that CENP-A delivery to centromeric chromatin is coupled with the recruitment of the transcriptional machinery (Chen et al., 2015). In human and fly cells, CENP-A has been shown to associate with the histone chaperone, FACT, which has been implicated in promoting transcription through chromatin by all three RNA polymerases (Belotserkovskaya and Reinberg, 2004; Obuse et al., 2004; Foltz et al., 2006; Okada et al., 2009; Chen et al., 2015). FACT is enriched at the centromeres of fly, chicken, human, and mouse cells, and in flies and chickens, this complex has been shown to be required for CENP-A localization (Okada et al., 2009; Chan et al., 2012; Chen et al., 2015). Given the ability of FACT to invade the nucleosome, breaking strong DNA/octamer contacts (Hondele et al., 2013), one model is that FACT destabilizes the nucleosome, facilitating the passage of

the transcriptional machinery (Hondele and Ladurner, 2013), which in turn could evict placeholder $(H3\text{-}H4)_2$ tetramers to make room for the assembly of new $(CENP\text{-}A\text{-}H4)_2$ tetramers (Chen et al., 2015). In fungi, however, FACT does not appear to be directly required for proper CENP-A deposition, but instead is involved in preventing the ectopic incorporation of CENP-A (Choi et al., 2012; Deyter and Biggins, 2014). Thus, it is possible that the direct involvement of FACT in CENP-A deposition is shared only among complex organisms.

The transcriptional machinery associated with centromeres can produce transcripts that either are stable or can be detected in genetic backgrounds that prevent their rapid processing (Nagaki et al., 2004; Choi et al., 2011; Ohkuni and Kitagawa, 2011; Chan et al., 2012). Centromeric transcripts have now been detected in a myriad of systems ranging from species as diverse as maize and frogs. In some instances, centromere-derived RNAs have been shown to play a structural role in centromere or kinetochore integrity (Topp et al., 2004; Ferri et al., 2009; Chan et al., 2012; Carone et al., 2013; Quénet and Dalal, 2014; Rošić et al., 2014; Blower, 2016).

Transcription could contribute to CENP-A chromatin function by promoting histone H3/CENP-A exchange or by generating transcripts that interact with the CENP-A assembly machinery, aiding in its function. Whether or not the primary role of centromeric transcription is to contribute to CENP-A deposition, as opposed to producing functional RNAs, is unclear.

Knockdown of specific centromere-derived RNAs causes defects in centromeric chromatin function (Carone et al., 2013; Quénet and Dalal, 2014); on the other hand, transcripts are also produced from noncentromeric genomic locations that have acquired CENP-A, such as naturally occurring neocentromeres in humans (Chueh et al., 2009), de novo centromeres on human artificial chromosomes (Bergmann et al., 2011), and synthetic centromeres in *Drosophila* (Chen et al., 2015). These findings suggest that transcription-mediated chromatin reorganization is associated with centromere chromatin establishment and maintenance regardless of the genomic location. Given that these nonnative centromeres appear to function reasonably well, either any RNA (centromeric or not) produced during CENP-A deposition can perform structural functions or specific centromere-derived RNAs play a secondary role. Alternatively, endogenous centromeric RNAs could supply critical structural functions in trans to kinetochores assembled at artificial centromeres or to neocentromeres. The observation that centromeric RNAs derived from specific chromosomes localize and affect the kinetochore function of other chromosomes in both *Xenopus* and *Drosophila* (Rošić et al., 2014; Blower, 2016) is consistent with this model.

Experiments in fission yeast suggest that the critical property of centromeric DNA lies in the particular transcriptional environment it provides for CENP-A loading. The central domain of the fission yeast centromere contains several transcriptional start sites and cryptic promoters from which low levels of transcripts are produced despite high levels of RNA polymerase II, consistent with transcriptional stalling. Mutants that increase stalling of RNA polymerase II augment CENP-A deposition, suggesting that the quality of transcription provides an optimal transcriptional environment for the correct amount of CENP-A to be deposited (Catania et al., 2015). Collectively, much of these recent findings, together with the notion that centromeric DNA evolves rapidly, raise the possibility that the main evolutionary constraint at centromeres is their amenability to moderate levels of transcription (Jiang et al., 2003; O'Neill and Carone, 2009; Chan and Wong, 2012; Catania et al., 2015). Separating the role of transcription itself from that of the resulting centromere-derived RNAs will be an exciting future challenge for the centromere biology field.

Conclusions and future directions

Since the discovery of CENP-A more than 30 years ago, our understanding of the mechanisms of its faithful deposition at centromeres has increased significantly. Major advances include the high-resolution imaging of chromatin fibers and intact nuclei with labeled histones, which has provided unprecedented spatial and temporal resolution of CENP-A dynamics through the cell cycle; the biochemical and structural studies that clarified the mechanics of $(CENP\text{-}A\text{-}H4)_2$ tetramer formation; and the dissection of the complex interplay between the CENP-A recruitment machinery involving Mis18 and the cell cycle. Yet, many key questions remain unanswered, for example, how transcription contributes to CENP-A chromatin establishment.

The CENP-A nucleosome assembly pathway harnesses the functions of histone chaperones, remodeling factors, transcription factors, and histone-modifying enzymes that play critical roles in maintaining chromatin integrity at noncentromeric regions. Thus, determining how the multiple roles of these factors do not conflict with one another (e.g., by promoting H3.3 instead of CENP-A incorporation at the centromere in G1) will be another important future direction of research.

Acknowledgments

We apologize to those authors whose work we did not cite because of space limitations. We thank Leah Rosin and Ankita Chavan for helpful comments and suggestions.

Our work on centromeres is supported by National Science Foundation award MCB1330667 and National Institutes of Health grant GM108829.

The authors declare no competing financial interests.

Submitted: 2 May 2016
Accepted: 14 June 2016

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