SURVEY AND SUMMARY

Centromeric and ectopic assembly of CENP-A chromatin in health and cancer: old marks and new tracks

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ABSTRACT

The histone H3 variant CENP-A confers epigenetic identity to the centromere and plays crucial roles in the assembly and function of the kinetochore, thus ensuring proper segregation of our chromosomes. CENP-A containing nucleosomes exhibit unique structural specificities and lack the complex profile of gene expression-associated histone post-translational modifications found in canonical histone H3 and the H3.3 variant. CENP-A mislocalization into noncentromeric regions resulting from its overexpression leads to chromosomal segregation aberrations and genome instability. Overexpression of CENP-A is a feature of many cancers and is associated with malignant progression and poor outcome. The recent years have seen impressive progress in our understanding of the mechanisms that orchestrate CENP-A deposition at native centromeres and ectopic loci. They have witnessed the description of novel, heterotypic CENP-A/H3.3 nucleosome particles and the exploration of the phenotypes associated with the deregulation of CENP-A and its chaperones in tumor cells. Here, we review the structural specificities of CENP-A nucleosomes, the epigenetic features that characterize the centrochromatin and the mechanisms and factors that orchestrate CENP-A deposition at centromeres. We then review our knowledge of CENP-A ectopic distribution, highlighting experimental strategies that have enabled key discoveries. Finally, we discuss the implications of deregulated CENP-A in cancer.

INTRODUCTION

Proper duplication and segregation of our chromosomes are of paramount importance to ensure genome stability and the successful transmission of genetic information from mother to daughter cells during cell division. Formation of a kinetochore at the centromere and attachment of the spindle fibres arising from the centrosome to the kinetochore are crucial during the mitotic phase of the cell cycle. Precise assembly of the kinetochore complex at the centromere is determined by localization of Centromeric Protein A (CENP-A), a histone H3 variant that confers epigenetic identity to the centromere and ensures its faithful inheritance through generations (1–5). CENP-A containing nucleosome particles exhibit structural specificities that are essential for kinetochore function. In addition, CENP-A lacks the complex profile of gene expression-associated histone post-translational modifications found in canonical histone H3 and the H3.3 variant, harboring instead only a small set of modifications that ensures mainly mitosis-specific functions. CENP-A mislocalization into noncentromeric regions (hereby referred to as ectopic localization) resulting from its overexpression has the potential to generate ectopic kinetochores (6,7) or weaken native kinetochores (8), leading to chromosomal segregation aberrations and genome instability. Although ectopic CENP-A can be induced by its artificial overexpression (6,9–11),

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is also a feature of many cancers, suggesting that non-physiological incorporation of CENP-A may assist tumori-genesis (12–16). The recent years have seen great developments in the molecular characterization of the factors and mechanisms that orchestrate CENP-A deposition at native centromeres and ectopic loci. In addition, they have witnessed the solution of the CENP-A nucleosome structure to nearly atomic level and a transition from the original description of CENP-A upregulation in cancer to the observation of novel, heterotypic CENP-A/H3.3 nucleosome particles and the exploration of the phenotypes associated with the deregulation of CENP-A and its chaperones, including their impact on chromosomal stability.

In this review, we sum up the structural specificities of the CENP-A nucleosomes, the epigenetic features that characterize the centromeric chromatin and the mechanisms and factors that orchestrate CENP-A deposition at centromeres. We then review the current knowledge on the ectopic deposition of CENP-A, highlighting some of the experimental strategies that have enabled key discoveries. Finally, we discuss the implications of deregulated CENP-A in cancer cells and suggest novel avenues of investigation.

STRUCTURE OF THE CENP-A NUCLEOSOME

CENP-A, like conventional H3, exhibits a typical core histone organization, consisting of a structured histone fold domain, flanked by flexible N- and C-terminal tails (Figure 1A) (17,18). However, the primary sequence of CENP-A shares only about 50% amino acid identity with conventional H3. In addition, the comparison of CENP-A proteins from different species reveals great divergence both in the N- and C-terminal tails (19), with the latter nonetheless retaining a hydrophobic region that constitutes a key determinant in the interaction with CENP-C (19). (Figure 1A).

The main histone variants of the H3 family are H3.3 and CENP-A (20–22). H3 (Figure 1A) and H3.3 are modified post-translationally on multiple sites (23). These post-translational modifications (PTMs) play essential roles in various nuclear processes, including transcriptional activation and silencing, as well as mitosis (23–25). In contrast, CENP-A exhibits only a limited number of PTM sites and a reduced panel of modifications (Figure 1A) (26,27). This simplified PTM pattern may reflect either a minor role for PTMs in the function of CENP-A and/or the specific and time-restricted involvement of CENP-A in mitosis but not in the other phases of the cell cycle. Consistent with this latter notion, human CENP-A is phosphorylated at serine 7 (S7) at the onset of mitosis (28). Phosphorylated CENP-A then becomes the target of the phosphoserine-/threonine binding proteins 14–3–3, which act as specific mitotic 'bridges' to link phosphorylated CENP-A and CENP-C, enabling CENP-A centromeric chromatin to function as a platform in the assembly and maintenance of active kinetochores (28). Of note, S7 phosphorylation of human CENP-A is mitotic specific, since at the end of mitosis it is dephosphorylated (29). Interestingly, human CENP-A is also phosphorylated at S16 and S18 and this double phosphorylation is important for mitotic chromosome segregation. The phosphorylation of both serines persists throughout the cell cycle and is detected in soluble (prenucleoso-

mal) CENP-A, as well as in both asynchronous and mitotic nucleosomal CENP-A complexes (26). Mutational analysis of the CENP-A N-terminal tail shows that this double phosphorylation motif leads to stronger intranucleosomal interactions that reflect the formation of a salt-bridged secondary structure (26).

Various models have been proposed for the centromeric nucleosome structure, including tetrameric models composed of two molecules each of CENP-A and H4, or one molecule of CENP-A and one molecule of H2A, H2B and H4 (30). However, the view prevailing nowadays is that CENP-A forms a histone octamer containing two each of histones H2A, H2B, H4 and CENP-A with the DNA left-handedly wrapped around the histone octamer, as in canonical nucleosomes containing histone H3 (18,31). Furthermore, the evidence suggests that CENP-A functions mainly via the structural specificities it confers to the CENP-A nucleosome (32). Indeed, although the 3D overall organization the CENP-A nucleosome is very similar to that of conventional H3 (Figure 1B), the crystal structure reveals distinct features of the CENP-A nucleosome (18), namely: (i) a different L1 loop. Two highly conserved residues (R80 and G81) are inserted in the CENP-A L1 loop relative to conventional H3, allowing recognition and specific binding by CENP-N, a component of the constitutive centromere-associated network (CCAN) complex required for active kinetochore assembly (Figure 1B) (33); (ii) a smaller αN helix. The αN helix of CENP-A consists of only two turns, compared to three for H3. Of note, the αN helix of H3 is essential for the wrapping of the nucleosomal DNA ends around the histone octamer and the rigid angle formed between the entry/exit ends of the nucleosomal DNA (Figure 1C) (34); (iii) flexible nucleosomal DNA ends. Only 123 bp of DNA are wrapped around the CENP-A histone octamer, in contrast to the conventional nucleosome where the histone octamer organizes 146 bp; 13 bp at each end of the nucleosomal DNA are not 'visible' in the CENP-A crystal nucleosome structure (Figure 1C), suggesting highly flexible ends. Recent cryo-electron microscopy experiments and micrococcal digestion studies have shown that this was also the case in solution (35,36). The highly flexible DNA ends displayed by the CENP-A nucleosome interfere with the binding of the linker histone H1 to the nucleosome, allowing a more open configuration of the CENP-A chromatin (35,37). This, in turn, enables binding of the CCAN complex to the CENP-A chromatin (35). In addition, by inhibiting H1 binding, CENP-A flexible ends are expected to favor the formation of DNA:RNA hybrid (R-loops) at centromeres (38); (iv) a distinct hydrophobic COOH-terminus, which is required for recognition of the centromeric nucleosome by CENP-C (Figure 1A) (19). Together, these unique structural features of the CENP-A nucleosome are crucial to the assembly and maintenance of active kinetochores (39).

Recently, nucleosome particles harboring the heterotypic tetramer CENP-A-H4/H3.3–H4 were observed in cancer cells in culture (40). Subsequent work revealed that heterotypic CENP-A/H3.3 nucleosomes consist of octamers (CENP-A-H4, H3.3–H4 and two H2A–H2B dimers) that display physical properties of both conventional H3 nucleosomes and the CENP-A nucleosome (41). Indeed, in
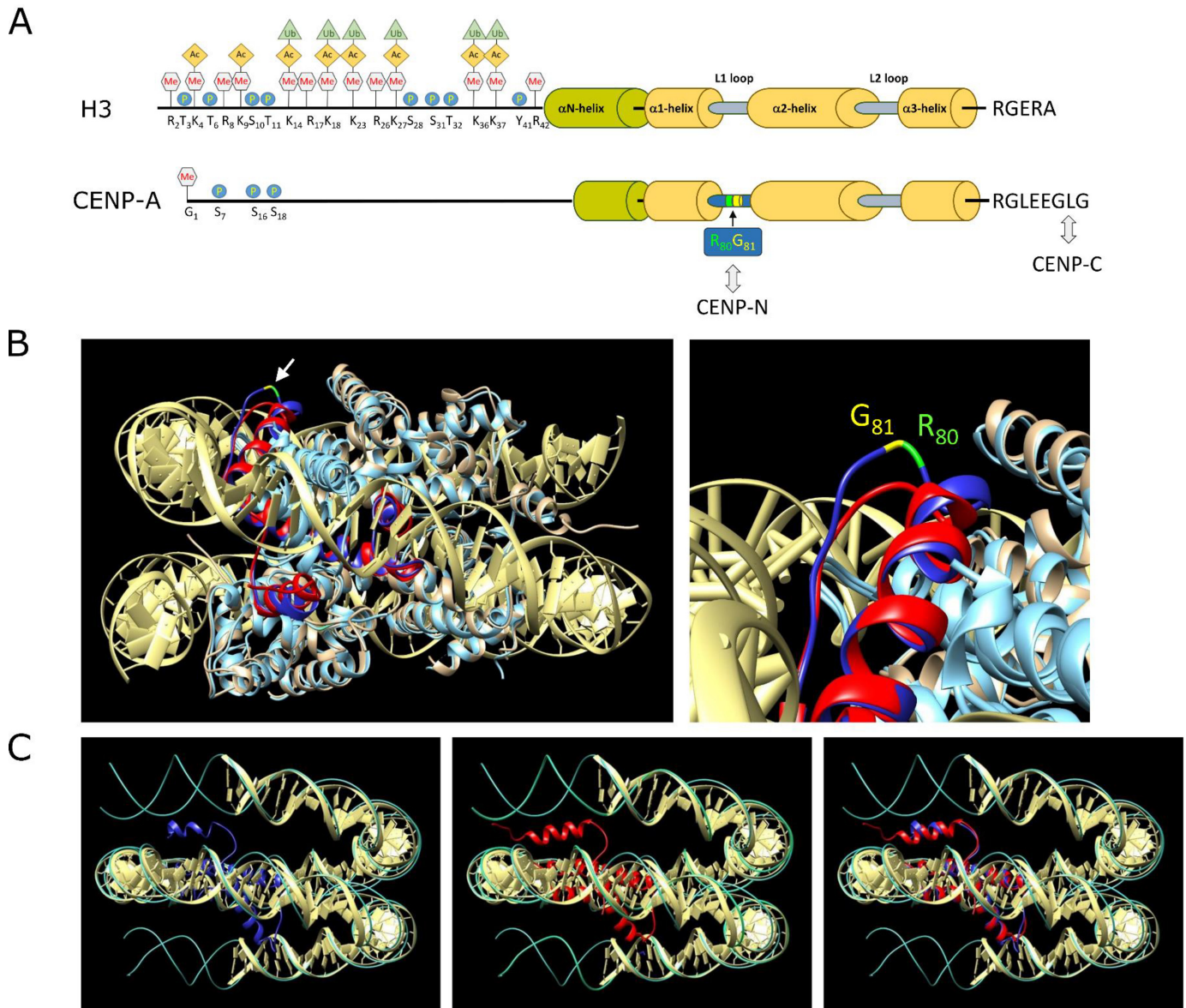
Figure 1. (**A**) Schematic representations of the secondary structure of CENP-A and conventional histone H3, together with a depiction of the sites and nature of the major posttranslational modifications known to occur on their N-terminal tail (P, phosphorylation; Me, methylation; Ac, acetylation; Ub, ubiquitination). The 2 amino acid insertion within the L1 loop of CENP-A (R80, G81) that mediates specific interaction with CENP-N, as well as its C-terminal end involved in interaction with CENP-C, are indicated. Note that the αN helix of CENP-A is one helical turn shorter than that of H3. (**B, C**). Salient differences between the CENP-A and H3 nucleosome structures. (**B**). Side view of the CENP-A nucleosome (PDB entry 3AN2) together with superimposed conventional core histone octamer (PDB entry 1KX5). The small loop containing CENP-A amino-acids R80 and G81 that protrudes from the surface of the nucleosome is indicated (arrow). The right panel shows an enlargement of the L1 loop area, with a superimposition of the L1 loops from CENP-A (blue) and H3 (red); residues R80 and G81 of CENP-A are shown in green and yellow, respectively. The core histones H2A, H2B and H4 are in light brown in the H3 octamer, while those of the CENP-A octamer are in light blue. (**C**). Side views of the superimposed DNA from CENP-A (yellow) and conventional H3 (light blue) nucleosome core particle together with either CENP-A (dark blue, left) or H3 (red, middle); The right panel shows a superimposition of DNA from CENP-A- and H3 core particle together with CENP-A and H3. Note that the nucleosomal DNA ends are not visible in the crystal of the CENP-A nucleosome and that the αN-helix of CENP-A exhibits two turns only.

line with the data for the CENP-A nucleosome particle, the DNA end close to CENP-A in the heterotypic particle appears more flexible than that close to H3.3. In addition, a single CENP-A R80/G81 mini-loop protrudes from the surface of the nucleosome, suggesting that each CENP-A/H3.3 nucleosome could interact with only one CENP-N molecule. Moreover, a single CENP-C protein was also found to interact with the CENP-A/H3 particle, consistent with the presence of only one CENP-A within the particle (41). Taken together, and since the N-terminal tail of H3.3, like H3, can undergo a complex pattern of PTMs at a very large number of sites (42), these observations suggest that the function and regulation of CENP-A/H3.3 heterotypic nucleosomes could involve both the specific structural features of CENP-A and PTMs associated with H3.3 and CENP-A. How these specificities contribute to the eti-

ology of cancers associated with CENP-A overexpression and altered distribution is discussed at the ultimate section of this review.

EPIGENETIC FEATURES OF CENTROMERIC CHROMATIN AND CENP-A ASSEMBLY

Genetic and epigenetic centromere specification

In the budding yeast *Saccharomyces cerevisiae,* centromeres are genetically defined by a ∼125-bp DNA sequence harboring a single, perfectly positioned CENP-A^{Cse4} nucleosome that covers the entire centromere (43). This short sequence, which is not flanked by pericentric heterochromatin, consists of three centromere-determining elements (CDEI-II-III) that collectively establish a functional centromere (44). Specifically, CDEI (a conserved 8-bp palindromic sequence) binds the transcription factor Cbf1, allowing transcription of the centromeric DNA (45–47), CDEII (a 78–86-bp AT rich sequence) is wrapped by CENP-A^{Cse4} (48), and CDEIII (a 26-bp partially palindromic sequence) is bound by the multiprotein complex CBF3 that bridges CDEIII to spindle microtubules (49,50).

The short 'point centromeres' of budding yeast contrast with the much larger 'regional centromeres' hosting multiple microtubule nucleation sites that are observed in most other eukaryotes, including fission yeast, *Drosophila* and humans. Regional centromeres show great differences in DNA sequence length, composition and organization, and are flanked by heterochromatin (51,52). For instance, fission yeast centromeres span 35–110 kb and are composed of a central core region (specifically, a central region (cnt/cc) of non-repetitive DNA surrounded by inverted repeat regions containing several tRNA genes (the innermost repeats, imr)) that contains several CENP-A^{Cnp1} nucleosomes, and flanking outer repeat (otr) elements that underlie heterochromatin (53). Although human CENP-A nucleosomes assemble on a small portion of the α-satellite (alphoid) locus, composed of highly divergent ∼171-bp tandem repeats units assembled in long multimeric higher-order arrays in a chromosome-specific manner (54,55), it can also load on neocentromeres that lack α-satellite repeats (9). In addition, while repetitive sequences compose the underlying DNA of centromeres in many species, these sequences are generally not conserved among species (44,56–59) and in many instances, centromeres display a mixture of repetitive and non-repetitive sequences (60–62). Thus, in regional centromeres CENP-A localization is not dictated by the sequence of the underlying DNA, but represents instead a defining epigenetic mark to establish the centromere-specific chromatin (63–65).

The centrochromatin

Conserved epigenetic marks at and around the centromere appear to play a significant role in ensuring the proper localization of CENP-A and the maintenance of centromere identity. The characterization of extended chromatin fibers has provided crucial observations on the unique pattern of histone PTMs - distinct from both euchromatin and heterochromatin - that characterizes the centromeric chromatin or 'centrochromatin', as initially coined by Sullivan and Karpen (66). CENP-A nucleosomes are confined to just a portion of an alpha-satellite array, forming a domain with finite size where CENP-A-containing nucleosomes intersperse with H3-containing nucleosomes (67). How such centromeric 'core' domains are regulated and their boundaries maintained is thought to involve multiple conventional core histone PTMs. In humans and *Drosophila*, centrochromatin maintains marks characteristic of actively transcribed regions (specifically, H3K4me2/H3K36me2) that are required for CENP-A assembly (66,68), whilst displaying low levels of other transcription-permissive histone PTMs (i.e., acetylated forms of histones H3 and H4, and H3K4me3) (69,70). Of note, studies with human artificial centromeres (HAC, described below) have shown that H3K9 acetylation levels at the centromere increase during a brief cell cycle window coinciding with the localization of the CENPA-chaperone HJURP (71). The evidence from chromatin fiber analyses suggests that centrochromatin is not associated with the heterochromatic marks H3K9me2/3 (66,68), although super-resolution microscopic analysis of chicken DT40 cells have revealed H3K9me3 within functional centromeres (72). In addition, recent analyses of human centromeric nucleosomal PTMs combining affinity purification and mass spectrometry have exposed the prominence of H3 nucleosomes containing the dual modification H3K9me2-K27me2, and also the enrichment of H4K20me1 in CENP-A-associated nucleosomes and adjacent H3 nucleosomes in cycling HeLa cells (73). This latter finding corroborates the observations made by Hori *et al.* (74) who also demonstrated that H4K20me1 is essential for kinetochore assembly. Finally, heterochromatin-associated histone methylation marks (e.g. H3K9me2/3 and H3K27me3) and heterochromatin protein 1 (HP1) are highly enriched in the regions that surrounds the centromere (66,67,69,73,75) where they help determine centromere boundaries (see below).

Deposition of CENP-A at the centromere

CENP-A chromatin plays a fundamental role in the assembly of the kinetochore and the building of the kinetochore-microtubule interface (44,52,72,76). Involved in this process in vertebrates is the constitutive centromere-associated network (CCAN), consisting of 16 distinct CENP proteins, that is specifically assembled onto centromeric chromatin (76,77). CCAN proteins are located in the inner kinetochore plate and distributed in several functional groups as follows: CENP-C, CENP-H/I/K/, CENP-L/M/N, CENP-O/P/Q/R/U, CENP-T/W and CENP-S/X. Some of these proteins, including CENP-B, CENP-C and CENP-T/W have DNA binding features. The main role of the CCAN is to recruit outer kinetochore components, such as KNL1, the Mis12 complex, and the Ndc80 complex (forming the KMN network) to which spindle microtubules with their structural and regulatory proteins will attach. Among the CENP members of CCAN, CENP-C and CENP-T are required in parallel for operational kinetochore specification and spindle attachment. As illustrated below, some

of these proteins also play a direct role in the deposition of CENP-A at the centromere. Of note, the complexity of CCAN observed in vertebrates contrasts with the situation in *Drosophila* where CENP-C is the only other constitutive centromeric protein in addition to CENP-A (78,79).

CENP-A undergoes replicative dilution during S-phase and is reintroduced in a cell cycle-restricted manner. Deposition of CENP-A occurs in early G1 in humans where it is mediated by the chaperone Holliday Junction Recognition Protein (HJURP) (80,81). A short and conserved N-terminal domain of HJURP, termed CBD (CENP-A binding domain), is required for the formation of the HJURP-CENP-A/H4 complex (82). CENP-A is deposited in centromeric chromatin in a sequence of reactions that involves CENP-C-mediated recruitment of the mitotic regulator complex Mis18 (composed of Mis18α, Mis18β and Mis18BP1) to the centromere (83,84), and the subsequent recruitment of HJURP by Mis18β, leading to targeted CENP-A assembly (85). Importantly, temporal regulation of Mis18 complex assembly through phosphorylation by Polo-like kinase 1 (Plk1) and cyclin-dependent kinase1 (Cdk1) restricts HJURP-mediated CENP-A deposition to early G1 (86–88). Reported data also suggest that CENP-A assembly can be mediated by CENP-I, independently of CENP-C, through the recruitment of Mis18BP1 (89). Other factors involved in the assembly of centromeric CENP-A chromatin in humans include the remodelling and spacing factor 1 (RSF1) (90) and the Rho family GTPase activating protein MgcRacGAP (91). In *Drosophila*, which lack a HJURP homologue, the assembly of CENP-A (called CID) is mediated by chromosome alignment 1 (CAL1), itself recruited to centromeres by CENP-C (92–95). When targeted to noncentromeric DNA (see below), both HJURP (96) and CAL1 (95) are able to recruit CENP-A/Cid and direct the assembly of a functional centromere. Finally, nucleosomes containing the histone variant H3.3 have been suggested to serve as 'placeholders' during S phase to ensure the subsequent assembly of CENP-A in G1 (97). The chromatin remodeling events that ensure the replacement of these 'placeholders' are currently unknown (98).

Transcription of centromeres and CENP-A loading

Consistent with the presence of permissive histone PTMs, studies in several organisms have demonstrated that centromeric DNA undergoes low levels of transcription by RNA polymerase II (RNA pol II) and there is an increasing amount of evidence to suggest that transcription of the underlying DNA into long non-coding RNAs (ncRNAs) is important for proper establishment of CENP-A chromatin and kinetochore function (99–107). In Drosophila, the characterization of an induced ectopic centromere system (described below) revealed that *de novo* CENP-A^{CID} assembly by CAL1 is coupled to RNA pol II-mediated transcription of the underlying DNA, in a process that involves recruitment of the histone chaperone Facilitates Chromatin Transcription (FACT) and RNA pol II by CAL1 (108). In addition, FACT depletion caused defective recruitment of CENP-A at endogenous centromeres with little effect on the retention of existing CENP-A, and resulted in the accumulation of H3.1 and H3.3 within centromeric chromatin. This led to a model whereby RNA pol II-mediated transcription causes eviction of the placeholders H3.1 and H3.3, allowing deposition of CENP-A^{CID} by CAL1 (108). In mammalian cells, RNA pol II-mediated transcription of CENP-A containing chromatin during mitosis and early G1 was shown to produce non-coding satellite transcripts important for stabilization of CENP-C (102), and recruitment of HJURP and CENP-A at the centromere (106). Recent work by Mc-Nulty *et al.* (103) indicates that human centromeric RNAs are produced and function in *cis*. Specifically, each human centromere produces a chromosome-specific set of ncRNAs that reflect the underlying α satellite sequence and organization and remain in distinct spatial domains throughout the cell cycle. Importantly, targeted depletion of distinct α satellite RNAs reduced the levels of CENP-A and CENP-C at the targeted centromere, leading to cell arrest prior to mitosis. Centromeric ncRNA transcripts arising from active α arrays were associated with CENP-A, CENP-B and CENP-C. Notably, transcription also occurred in inactive α arrays, which are enriched in repressive histone marks (109), but the resulting ncRNAs, which were only associated with the α satellite DNA binding protein CENP-B, were less stable than those produced at active arrays. As CENP-B can promote and maintain heterochromatin on ectopic α satellite (110), McNulty *et al.* (103) propose that CENP-B bound to ncRNAs arising from inactive arrays could promote pericentric heterochromatin assembly and maintenance, in contrast to its function in promoting CENP-A assembly and stabilization at active α-arrays, in the presence of permissive histone PTMs (71,110)

It has been proposed that centromeric transcription facilitates a transition from chromatin association to stable incorporation of CENP-A (104). However, it is worth to note that *in vitro* CENP-A chromatin was predominantly nonpermissive for transcription compared to H3 chromatin (111), suggesting that once stably incorporated, CENP-A itself could modulate transcription within centromeric chromatin. In addition, work by Molina *et al.* (112) indicates that centromeric transcription, promoted by H3K4me2 and associated with H3K9 acetylation, prevents the spreading of heterochromatin into the centromere. The authors also suggest that transcription inhibition resulting from the loss of H3K4me2 decreases CENP-A incorporation and the replacement of the H3.3 placeholders, in agreement with the findings in Drosophila (108).

Finally, studies suggest that non-centromeric RNA species may also play a role at centromeres. Thus, it is notable that SAT III satellite long ncRNAs have been found to localize to centromeric regions in Drosophila (104,113). SAT III ncRNAs bound to CENP-C and were required for correct localization of CENP-A and CENP-C, as well as outer kinetochore proteins, and for prevention of mitotic defects (104,113).

Non-B-form DNA, centromeric transcription and centromere specification

Non-B-form DNA conformations such as hairpins, singlestranded DNA, triplexes, R-loops and i-motifs have been observed at centromeres in a variety of organisms including human (114–118), suggesting that DNA-encoded structural

features may contribute to centromere specification. Recent analyses by Kasinathan and Henikoff (119) have provided new insights into this notion, revealing that in certain organisms such as old world monkeys, chicken and yeast, functional centromeres were characterized by the presence of short (<10 bp) DNA dyad symmetries predicted to adopt non-B-form DNA, which correlated with the absence of CENP-B boxes (120) and, accordingly, CENP-B binding. In contrast, the α arrays of functional centromeres in organisms such as great apes and mice globally lacked dyad symmetries, yet displayed non-B-form DNA as well as CENP-B boxes bound by CENP-B. Notably, greater enrichment for non-B-form DNA was detected at α satellite sequences associated by CENP-B boxes and occupied by CENP-A. In addition, native centromeres lacking CENP-B boxes (e.g. the centromere of human Y chromosome) were enriched in DNA dyad symmetries and non-B-form DNA structures.

As HJURP displays the ability to bind synthetic holliday junctions in vitro (121), the authors suggest that, in organisms such as old world monkeys, stable cruciform DNA structures forming spontaneously in centromeric satellite DNA may be recognized by HJURP or its orthologs, leading to CENP-A deposition. Instead, in organisms such as great apes, the formation of cruciform structures attracting HJURP may be facilitated by CENP-B binding, for instance as a result of DNA bending induced by CENP-B (122). In a non-exclusive alternative model that takes into account the requirement for transcription in CENP-A assembly, these authors propose that transcription of satellite DNA may occur readily at centromeres that adopt non-B-form DNA or instead require DNA bending mediated by CENP-B. While these models require testing, it is worth noting that Kabeche *et al.* (123) have recently reported the presence of R-loops at centromeres in human cells. These R-loops, generated through RNA pol II transcription during mitosis, are involved in a mitosis-specific Ataxia Telangiectasia Mutated and Rad3-Related (ATR)-driven signalling pathway that promotes accurate chromosome segregation. Interestingly, the authors also showed that ATR and CENP-B colocalized at centromeres. It remains to be determined whether centromeric R-loop formation requires CENP-B and how such structures contribute to HJURP recruitment at the centromere.

Proteolysis and the control of CENP-A deposition

Several reports suggest that even when CENP-A is expressed at endogenous levels, its loading in early G1 takes place not only at or near existing sites of centromeric CENP-A-containing chromatin but also at non-centromeric, ectopic sites (31,40) (bioRxiv doi: https://doi.org/10.1101/428557). Given that ectopic CENP-A causes genetic instability (see below), it is notable that several organisms have evolved mechanisms relying on proteolysis to ensure the strict control of CENP-A protein levels and prevent its ectopic deposition. In budding yeast, CENP-A^{Cse4} levels are controlled by ubiquitylation mediated by the Psh1 E3 ubiquitin ligase and subsequent proteolysis (124,125). Thus, overexpression of CENP-A^{Cse4} is toxic in cells lacking Psh1 and results in ectopic CENP-A^{Cse4} localization (126). Proteolysis of CENP-A^{Cse4} is pro-

moted by phosphorylation of Psh1 mediated by the Cka2 subunit of casein kinase 2 (CK2) (127), and facilitated by histone H4, with H4-K36 promoting the interaction between Psh1 and CENP-A^{Cse4} (128) as well as the ubiquitin binding protein Doa1 (129). Importantly, Mishra *et al.* (130) have determined that centromere-associated CENP-A^{Cse4} was protected from Psh1-mediated ubiquitination by Pat1 (protein associated with topoisomerase II). Additional factors involved in CENP-A^{Cse4} degradation include the peptidyl-prolyl cis-trans isomerase Fpr3 (131) and the F-box containing protein Rcy1 (132). Recently, CENP-A^{Cse4} degradation via the SUMO-targeted ubiquitin ligase Slx5 was proposed as a Psh1-independent mechanism preventing CENP-A^{Cse4} mislocalization and genome instability (133).

Regulation of CENP-A levels through proteolysis was also reported in fission yeast (134), as well as in *Drosophila* (135) where it involved interaction of Ppa (F box protein partner of paired), a subunit of the SCF E3 ubiquitin ligase complex, with the CATD domain of CENP-A^{Cid} (136). Interestingly, the SFC complex was inactive in anaphase, when CENP-A^{Cid} is deposited at centromere. The authors have suggested that SCF-mediated proteolysis could be a general mechanism for regulating the levels and spatial distribution of CENP-A at the centromeres.

Although CENP-A proteolysis can be induced by the herpes simply virus type 1 ICP0 protein in infected human cells (137), regulation of CENP-A levels and localization through proteolysis has hitherto not been reported in mammalian cells. Whether alternative mechanisms take place in these cells remains to be investigated. Notable in this regard is a recent study by Nechemia-Arbely *et al.* (bioRxiv doi: https://doi.org/10.1101/428557) showing that endogenous CENP-A loaded ectopically onto thousands of ectopic sites in transcriptionally active chromatin in early G1 is removed in S phase by a DNA synthesis-mediated error correction mechanism that helps maintain an epigenetically defined centromere identity.

CHROMATIN MODIFIERS AND CENTROCHROMATIN DYNAMICS

Although still fragmentary, our knowledge of the epigenetic mechanisms that operate to facilitate CENP-A deposition and kinetochore formation has greatly benefited from epigenetic engineering experiments. In *Drosophila*, these include the specific targeting of centromeric factors and chromatin modifiers expressed as fusion to the Lac repressor (LacI), to DNA arrays of the Lac operator (LacO) ectopically-integrated in the chromosomes (Figure 2A) (10,96,138). In human cells, ectopic alphoid sites integrated at distinct chromosomal sites and human artificial chromosomes (HAC) - both resulting from the transfection of alphoid DNA arrays, offer powerful investigation tools. These include synthetic alphoid DNA arrays that incorporate regulatory elements such as tetracyclin operator (tetO) sequences (alphoid^{tetO}), thus allowing the tethering of centromeric and chromatin factors expressed as tetracyclin repressor (tetR) fusions (Figure 2B) (139,140). Some of the key findings made using these tools are presented below.
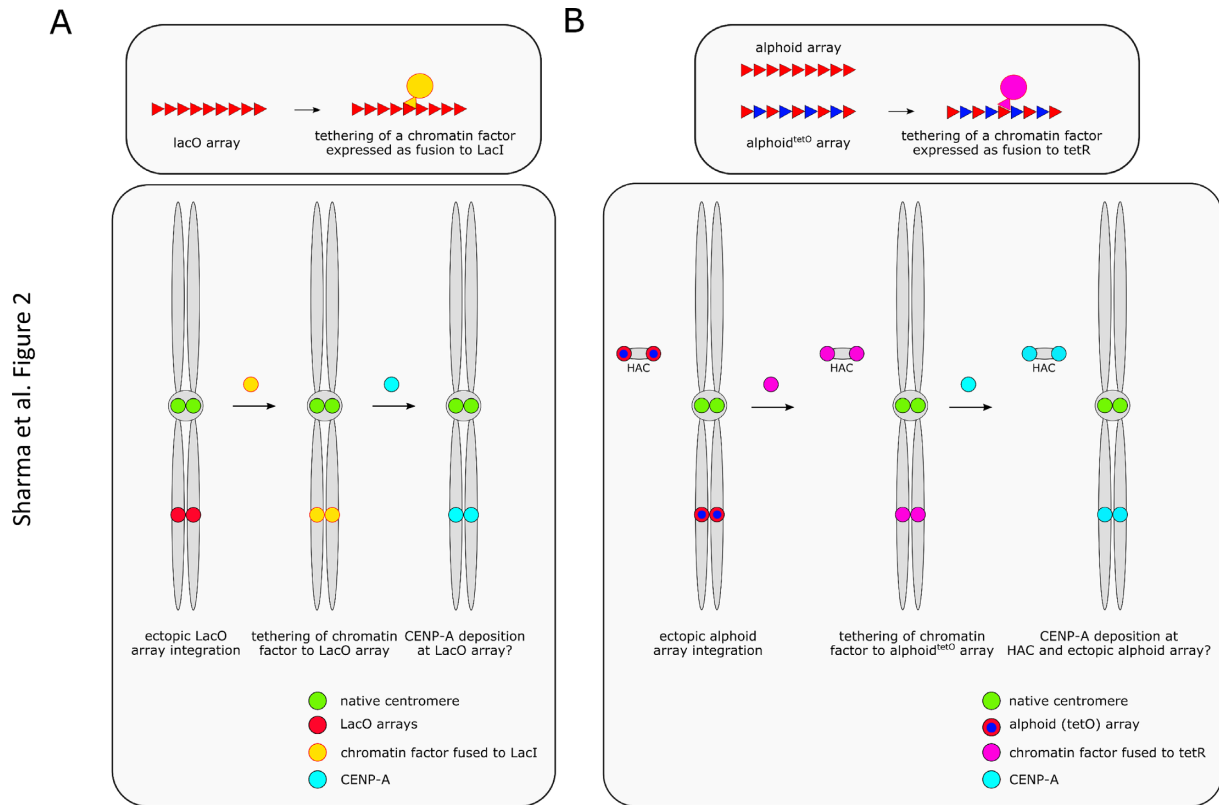
Figure 2. Artificial and natural systems for the study of CENP-A ectopic deposition. (**A**). Illustration of the lacO/lacI tethering system developed in *Drosophila*. The upper panel depicts an array composed of multiple copies of the lac operator (lacO)(red triangles), allowing the tethering of centromeric/chromatin factors fused to the lac repressor (lacI). Introduction of plamids bearing such an array into flies leads to their stable ectopic integration (lower panel). Note that an inducible centromeric/chromatin factor-LacI fusion construction is often used, which allows the time windows of chromatin changes to be monitored. (**B**). Illustration of the synthetic, alphoid^{tetO} technology. The upper panel depicts an alphoid^{tetO} array derived from α satellite repeats (red triangles) by integration of tetO sequences (blue triangles), allowing tethering of chromatin factors expressed as tetR-fusion proteins. When inserted into a cloning vector and transfected into cells, such arrays can integrate ectopically into a chromosome arm or form a human artificial chromosome (HAC)(lower panel). Whereas ectopic alphoid^{tetO} array integration enables dissection of the chromatin factors and states that promote CENP-A deposition, the characterization of HACs also allows de novo centromere and kinetochore assembly to be explored. Beyond the scope of this review, it should be noted that *de novo* HAC formation requires the presence of CENP-B-box sequences in the α satellite DNA (not shown here) (reviewed by Molina *et al.* (112). Variations on the technology oulined here include the simultaneous tethering of factors with antagonist chromatin modifying activities Molina *et al.* (140).

Heterochromatin boundaries

The significance of heterochromatin boundaries flanking centromeres has been extensively studied in fission yeast and *Drosophila*.

Using fission yeast as a model system, Folco *et al.* (141) showed that flanking heterochromatin directed by RNA interference (RNAi) was required for de-novo incorporation of CENP-A^{Cnp1} and kinetochore assembly over the central domain of a centromere lacking CENP-A^{Cnp1} (141). Heterochromatin, however, was not required for further propagation of the assembled CENP-A^{Cnp1} chromatin. Further work by this group determined that synthetic heterochromatin formation at euchromatic loci, via tethering of Clr4 (the *Schizosaccharomyces pombe* homolog of Su(var)3–9), enabled de-novo CENP-A^{Cnp1} incorporation and functional kinetochore assembly regardless of active RNAi (142). Interestingly, Yang *et al.* (143) have recently shown that *S. cerevisiae* CENP-A^{Cse4} can localize to centromere in fission yeast and partially substitute for CENP-A^{Cnp1}. Although overexpression of CENP-A^{Cse4} re-

sulted in ectopic deposition in *S. pombe*, proteolysis ensured low expression levels. Furthermore, association of CENP-A^{Cse4} with centromeres in fission yeast was dependent on flanking heterochromatin and the RNAi machinery, and RNAi-dependent pericentric heterochromatin enabled centromeric localization of Cse4 (and also Cnp1) by protecting these proteins from ubiquitin-mediated degradation. Whether similar mechanisms involving pericentric heterochromatin-mediated protection of centromeric CENP-A occur in other organisms remain to be investigated.

In *Drosophila*, local induction of heterochromatin formation through tethering of the heterochromatin protein HP1 caused the proximal deposition of ectopic CENP-A (10). Given the interplay between HP1 (which binds H3K9me2/3) and the histone methyltransferase Su(var)3–9 that deposits these marks (144–148), it will be illuminating to learn whether ectopic CENP-A assembly under these conditions results from an interaction between HP1 and Su(var)3–9. However, these observations give a measure of

the importance of the repressive nature of the centromere-bordering heterochromatin in CENP-A assembly.

Chromatin dynamics at the centromere

Related experiments have shown the deleterious impact of heterochromatin nucleation within the centromere, revealing that contrary to the surrounding heterochromatin, CENP-A assembly and kinetochore function are regulated by a complex balance between histone H3K9ac and H3K9me3. Thus, in human cells, tethering of SUV39H1 (the homologue of *Drosophila* Su(var)3–9) to the alphoid site of a human artificial chromosome (alphoid-HAC) was associated with increased H3K9me3 marks and prevented *de novo* CENP-A assembly and kinetochore formation (71). This observation corroborated previous studies indicating that tethering of HP1 (139) or the Kruppel-associated box (KRAB)-associated co-repressor KAP1 (149) induced the loss of CENP-H, CENP-C and CENP-A, eventually inactivating the kinetochore. In contrast, alphoid-HAC tethering of the histone acetyl transferase (HAT) domains of p300 or the p300/CBP associated factor PCAF—two HATs normally found at functional centromeres, resulted in the transient acetylation of H3K4 during a time window that coincided with MIS18α presence at the centromere, allowing the establishment of CENP-A chromatin and novel kinetochore formation in G1 (71). Importantly, HAT-mediated CENP-A assembly was dependent upon HJURP, but it bypassed the need for Mis18α (71). Subsequent work by the same group (150) revealed an interaction between the Mis18 subunit Mis18BP1 and the acetyltransferase KAT7/HBO1/MYST2, and showed that the KAT7-Mis18 complex also included HJURP and recruited RSF1 to the alphoid DNA. CRISPR/Cas-mediated KAT7 depletion reduced CENP-A localization at centromeres, whereas tethering of KAT7 (and also that of RSF1) to an ectopic alphoid DNA array lacking detectable CENP-A and harbouring high H3K9me3 levels led to a reduction of the H3K9me3 levels associated with new CENP-A assembly. The authors thus suggested that Mis18 recruits KAT7 to the centromere to prevent SUV39H1-mediated heterochromatin invasion within the centromere, by mechanisms that include both histone acetylation and loss of H3K9me3 marks through histone turnover/exchange promoted by RSF1.

Likewise, tethering the H3K4 demethylase LSD1 promoted H3K4me2 (and also H3K36me2) depletion and abrogated local transcription, resulting in decreased incorporation of newly-synthesized CENP-A (68). Importantly, targeting of LSD1 abolished HJURP recruitment at the HAC centromere, suggesting that the chromatin state and/or transcription of centromeres regulates the targeting of the CENP-A deposition machinery, hence the efficiency of CENP-A deposition (68). More recently, Martins *et al*. (75) have investigated how centromeres can resist silencing promoted by flanking heterochromatin marks to maintain local transcription with their results suggesting that the centrochromatin is able to influence the ability of epigenetic readers to translate local PTMs into silent chromatin states. Thus, centromeres resisted transcriptional repression associated with EZH2-mediated H3K27me3 deposition whereas such resistance could be bypassed by di-

rect tethering of the downstream repressor, Polycomb Repressive Complex 1 (PRC1). Likewise, unlike tethering of HP1 or full-length SUV39H1, tethering of a construct containing only the C-terminal H3K9 methyltransferase domain of SUV39H1 (i.e. lacking the H3K9me3-binding domain as well as regions required for interaction with HP1 and histone deacetylases) failed to induce HAC centromere inactivation. Although the underlying mechanisms remain to be elucidated, it is tempting to speculate that they involve RSF1-mediated removal of H3K9me3 marks before they can be interpreted by HP1. In addition to such chromatin remodelling mechanisms, demethylation of transcriptional silencing marks by centromere-linked histone demethylase could occur to maintain centrochromatin H3K9me2 and H3K36me2. Thus, sumoylation of origin recognition complex 2 (ORC2) in the G2/M phase was shown to recruit KDM5A to the centromere, promoting the conversion of H3K4me3 into the transcription permissive mark H3K4me2 (151). Notably, loss of ORC2 sumoylation resulted in H3K4me3 accumulation, decondensation of pericentric heterochromatin and chromosome instability at the G2/M phase. The role of other demethylases such as the H3K9/K36me3 lysine demethylase KDM4A/JMJD2A (152) at the centromere remains to be investigated.

Finally, using human cell lines harboring an alphoid-HAC, or an ectopic alphoid^tetO array devoid of CENP-A and integrated in a region covered with heterochromatin, Shono *et al*. (89) carried out a screen of centromeric factors and chromatin modifiers to identify the factors that influence CENP-A assembly. Their studies led to the identification of 4 classes of factors, with class I factors increasing endogenous CENP-A assembly on the HAC centromere and inducing *de novo* CENP-A assembly at the ectopic site, class II factors increasing endogenous CENP-A assembly on the HAC only, class III factors inducing efficient *de novo* CENP-A assembly to the ectopic site provided that CENP-A was overexpressed, and class IV decreasing endogenous CENP-A assembly on the HAC and preventing *de novo* CENP-A assembly at the ectopic site.

One interesting observation one can make from this classification is the contrasting position of the histone H3K9 methyltransferases G9a (class III) and SUV39H1 (class IV). Indeed, SUV39H1 and G9a play distinct roles in maintaining constitutive (e.g. pericentric) and facultative H3K9 methylation, respectively (153,154). In addition, G9a is a major H3K9me2 histone methyltransferase in mammalian cells. The observations by Shono *et al*. (89) therefore suggest a positive role for G9a, but not SUV39H1, in facilitating de novo CENP-A assembly in human cells, through the establishment of H3K9me2. Of note, the relative contribution of the *Drosophila* homologues of SUV39H1 (Su(var)3–9 (155) and G9a (dG9a/EHMT2) (156,157)) to ectopic CENP-A deposition remains to be determined. Recent evidence obtained from the study of RNAi-associated heterochromatin formation at pericentric DNA repeats in fission yeast by Jih *et al*. (158) indicates that contrary to H3K9me3 domains which are transcriptionally silent, H3K9me2 domains are involved in the formation of a transcriptionally permissive heterochromatin that allows RNAi-dependent co-transcriptional gene silencing at the pericentric DNA repeats. In addition, H3K9me2 precedes H3K9me3 dur-

ing heterochromatin establishment and the transition from H3K9me2 to H3K9me3 (for which HP1 shows greater affinity) is required for subsequent RNAi-independent, HP1-dependent epigenetic inheritance of H3K9 methylation. Based on these findings, we propose that H3K9me2 (which shows faster kinetics of establishment than H3K9me3 (158,159)) helps to ensure a transcriptionally permissive chromatin state within the centromere core. In addition, yet-to-be-identified mechanisms must operate to prevent the transition from H3K9me2 to H3K9me3 and the establishment of a heterochromatin state that would otherwise be deleterious to CENP-A assembly and kinetochore function. Such mechanisms may involve active histone demethylation or be related to those proposed by Martins *et al.* (75) to explain why centromeres resist transcriptional repression mediated by EZH2-mediated H3K27me3 deposition but not direct tethering of the downstream reader/repressor PRC1. Thus, fast removal of H3K9me3, like that of H3K27me3, would help prevent heterochromatin formation. Furthermore, although not addressed experimentally by Martins *et al.* (75), the balance between H3K27me2 and H3K27me3 may also be important for proper centrochromatin dynamics. H3K27me2/3 marks are deposited primarily by the EZH1 and EZH2 components of the Polycomb Repressive Complex 2 (PCR2) (160) and, similarly to H3K9 methylation, H3K27me2 is formed with a higher efficiency than H3K27me3 (161,162). Whereas H3K27me3 is associated with transcriptional repression, the role of H3K27me2 is more obscure. However, the evidence gathered in embryonic stem cells (ESCs) indicates that H3K27me2 helps prevent inappropriate transcription (162). In addition, H3K27me3 and H3K27me2 mark different regulatory regions in ESCs, with H3K27me3 mainly enriched at regions flanking transcription start sites whereas H3K27me2 accumulates in regions characterized by the presence of DNase I hypersensitive sites (163). Whether H3K27me2 could play a role similar and/or complementary to that of H3K9me2 in promoting centrochromatin formation remains to be learned. However, it is interesting to note that, in ESCs, H3K27me2 is found colocalizing with H3K36me2 as this latter mark is abundant within centromeres and di-methylation of H3K36 helps prevent H3K27me3 accumulation in ESCs (164). Although the relative contribution of H3K9me2/me3 and H3K27me2/me3 to centromere assembly and function awaits further investigation, it is striking that centromeres contain H3 nucleosomes with the dual modification H3K9me2-K27me2 (73) and that G9a can mediate not only H3K9me2 but also H3K27me2 (165–168). A schematic summary of the chromatin states and factors that mediate CENP-A deposition at centromeres is presented in Figure 3, together with the major histone PTMs that mark the centrochromatin and flanking pericentric heterochromatin.

ECTOPIC LOCALIZATION OF CENP-A

Recruitment to DNA double stranded breaks

CENP-A, together with CENP-C and CENP-T helps maintain centromeric α-satellite repeats integrity in human cells (169). Beyond the scope of this review, recruitment of CENP-A to DNA double stranded breaks (DSBs) induced

by laser or site specific endonuclease has been described (170), suggesting a role for CENP-A in DNA repair. Of note, the recruitment of CENP-A to the DSB was a slow process taking several minutes (170), while the recruitment of repair proteins is a very rapid process completed in sub-minute periods of time (171). In addition, although more recent experiments using multiphoton laser irradiation observed the recruitment at DSBs, of RSF1, which in turn facilitated recruitment of CENP-S and CENP-X, little or no CENP-A recruitment was observed under these conditions (172). Even though overexpression of CENP-A leading to its ectopic distribution conferred resistance to DNA damage (40) (see below), the contribution of CENP-A to DNA repair through its possible recruitment at sites of DNA damage thus remains an unresolved question.

### Overexpressed CENP-A and ectopic kinetochore formation in humans and flies

CENP-A overexpression and its ectopic localization have been observed in many cancer types, including colorectal and breast cancers (12,14,173), and extensively documented using the *Drosophila* and yeast model systems where over-expression of CENP-A alone leads to its ectopic localization and the assembly of functional kinetochores (6,174). In contrast, ectopic CENP-A localization due to its artificial overexpression does not result in ectopic kinetochore formation in mammalian cells (9,40). Instead, the evidence suggests that ectopic kinetochore formation in human cells requires simultaneous overexpression of CENP-A and CENP-C (175). This observation may reflect the complexity of the CCAN network in human, compared to *Drosophila*.

Ectopic CENP-A deposition and the H3.3 chaperone DAXX

Different histone chaperones ensure the replication-independent deposition of histone H3.3 (176,177), including DAXX and ATRX which are involved in deposition of this histone variant at pericentric heterochromatin and telomeres (178–180). Notably, RNAi studies in HeLa cells stably overexpressing CENP-A have revealed that, in humans, ectopic localization of CENP-A depended essentially on DAXX instead of HJURP, whereas depletion of ATRX affected the extent of ectopic deposition (40). Interestingly, overexpression of CENP-A afforded increased resistance to DNA damage induced by camptothecin or ionizing radiation. The association of ectopic CENP-A with DAXX and ATRX was also underlined in studies of colorectal cancer cell lines, where CENP-A is commonly overexpressed (12). Of note, increased CENP-A levels correlated with the upregulation of DAXX and ATRX, but not HJURP, in these cells (12) (but see also below our discussion of Filipescu *et al.* (181)). It is at present unknown whether ectopic deposition of CID in *Drosophila* involves a similar switch in chaperone usage.

Sites of ectopic CENP-A nucleosome assembly

Several investigations have shed light on the identity and features of the regions affected by ectopic CENP-A assem-
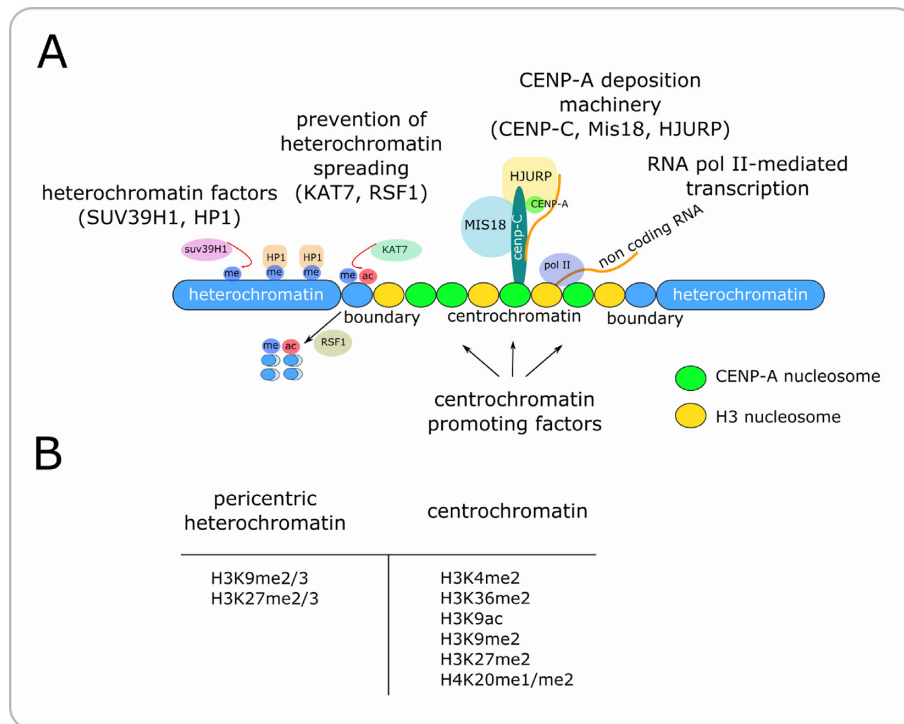
Figure 3. (**A**) Salient features of CENP-A deposition at the centrochromatin. Shown is a schematic summary of the CENP-A chaperone and deposition machinery including non-coding RNA originating from the underlying DNA. Maintenance of a boundary between centrochromatin and flanking pericentric heterochromatin domains (hereby illustrated by the SUV39H1/HP1-mediated deposition of H3K9me2/3) involves prevention of pericentric heterochromatin spreading into the centromere, which has been proposed to involve histone acetylation orchestrated by KAT7 and also histone turnover reactions mediated by the chromatin remodeler RSF1. KAT7-recruited RSF1 has also been suggested to promote CENP-A assembly directly. Not shown here are the histone epigenetic marks, turnover mechanisms and responsible chromatin factors that define the centrochromatin signature and impact its dynamics. These include a short window of H3K9 acetylation that accompanies Mis18/HJURP localization at the centromere, as well as histone modifications that contribute to a transcriptionaly-competent chromatin state. (**B**) Histone post-translational modifications that mark the centrochromatin and its flanking pericentric heterochromatin. Note that the H3K9me2 and H3K27me2 marks have been proposed to occur predominantly as a dual H3K9me2-K27me2 modification at the centromere.

bly. Studies in fission yeast have revealed the ectopic assembly of overexpressed CENP-A within centromeric outer repeats and pericentromeric regions, as well as rDNA repeats and the intergenic regions of subtelomeric domains (11,182). Telomere repeats alone could promote nearby CENP-A incorporation when inserted at an internal locus, and such repeats showed a high level of H3K9me2. Importantly, the assembly of CENP-A near the telomeres of synthetic minichromosomes required the telomere binding protein and modulator of telomerase activity Ccq1 as well as deposition of H3K9me2 by the histone methyltransferase Clr4, itself recruited by Ccq1, a finding that further supports the notion that H3K9 di-methylation may create favourable conditions for the ectopic localization of CENP-A. However, when natural chromosomes (including natural telomeric regions) were considered, ectopic CENP-A could assemble neocentromeres (albeit at reduced levels) independently of heterochromatin, suggesting alternative assembly routes (182).

In *Drosophila*, ectopic CID was found preferentially in regions bordering telomeres and pericentric heterochromatin, in transcriptionally silent intergenic chromatin domain (10). In human cancer cells and tumors, Athwal *et al.* (12) identified ectopic CENP-A hotspots at subtelomeric and pericentromeric regions, and also accessible chromatin domains at

gene promoters and transcription factor sites. These observations corroborate earlier studies in HeLa cells artificially overexpressing CENP-A, showing that ectopic CENP-A mapped not only to α-satellite regions but also to regions containing other DNA repeats or non-repetitive sequences, and that ectopic CENP-A hotspots included active regulatory elements (such as active enhancers, i.e. enriched in H3K4me1 and H3K2ac, promoters, transcription factor binding sites and binding sites for the insulator protein CTCF) (40).

Importantly, whereas CENP-A nucleosome particles are essentially homotypic (31,40) at the centromere, those at ectopic position were made up of both CENP-A/CENP-A homotypic and CENP-A/H3.3 heterotypic particles. In addition, specific positioning of heterotypic particles was observed at CTCF binding sites and active enhancers, which are sites of high histones turnover normally enriched for H3.3 and H2AZ (40). Given that H3.3 marks the pericentric and (sub)telomeric heterochromatin (176,179,183–186) as well as active promoters and gene regulatory regions (187–189), and that H3.3 has been proposed to act as 'placeholder' for the assembly of CENP-A at the centromere (97), it remains to be learned whether the preferential deposition of overexpressed CENP-A to these ectopic sites and the for-

mation of heterotypic particles reflect the interplay of dynamic H3.3 nucleosomes and DAXX.
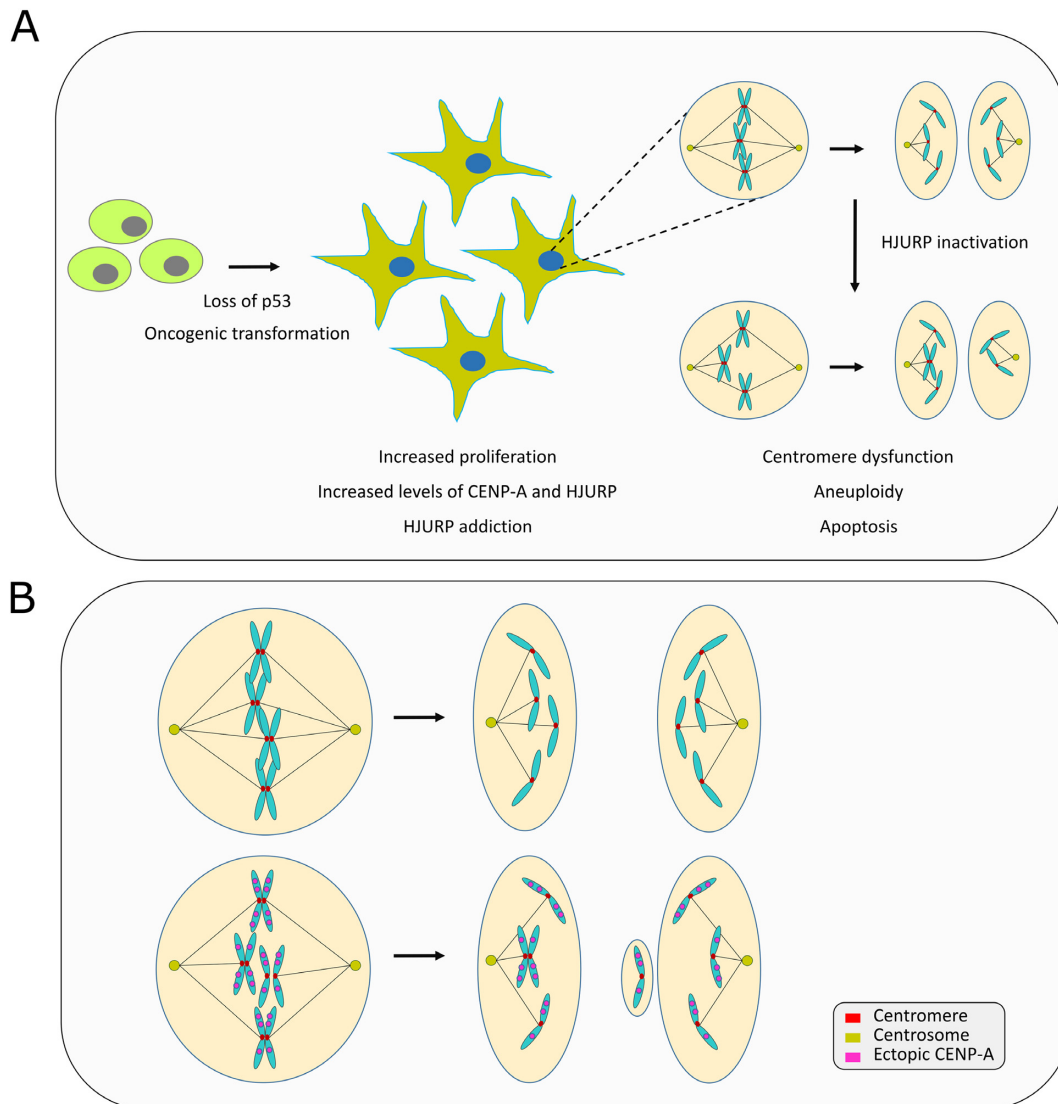
CENP-A DEREGULATION AND CANCER

Overexpression and ectopic assembly of CENP-A are observed in several cancers (7,13,14,16,173,190–194). Importantly, elevated CENP-A expression correlates with malignant progression and poor patient outcome in several cancer types (13,16). Presence of CENP-A in homotypic or heterotypic nucleosomes is expected to alter the pattern of methylation and acetylation PTMs associated with H3 and H3.3. Since hotspots for ectopic CENP-A deposition include accessible gene regulatory regions and transcription factor binding sites (12,40), and given that CENP-A can interfere with transcription *in vitro* (111), the issue arises as to whether the presence of CENP-A at noncentromeric locations impacts tumorigenesis by affecting gene expression. In this regard, it is notable that in HeLa cells artificially overexpressing CENP-A, its ectopic localization did not disrupt significantly the transcriptome (40). Whether ectopic CENP-A, possibly in combination with the deregulation of other chromatin/centromeric factors (12,13), affects the etiology of some cancers via a direct impact on gene expression remains unsolved.

Documented phenotypes of overexpressed CENP-A in cancer cells include its accumulation within the 8q24/Myc subtelomeric region long associated with genomic instability (12). Moreover, CENP-A deregulation has a crucial impact on chromosomal instability (CIN), a predominant hallmark of cancer characterized by chromosome missegregation (leading to aneuploidy) and rearrangements resulting from aberrant centromere and kinetochore function (7,8,195–197). Filipescu *et al*. (181) found that both CENP-A and HJURP were overexpressed in tumors carrying p53-inactivating mutations, resulting in the stabilization of the histone/chaperone complex and increased CENP-A deposition to centromeres. Parenthetically, proper CENP-A deposition could also be observed in HJURP-overexpressing cells, whereas both centromeric and ectopic deposition were seen in cells exogenously overexpressing CENP-A only, further stressing the specific role of HJURP in mediating centromere-specific deposition of CENP-A and the need for proper dosage between CENP-A and its chaperone. The authors also established, through the characterization of a primary mouse embryonic fibroblast (MEF) model that CENP-A and HJURP underwent further upregulation upon combined loss of p53 and oncogenetic transformation, thereby enabling the maintenance of a hyperproliferative state. Indeed CRISPR/cas9-mediated HJURP depletion in these cells resulted in loss of proliferation, centromere dysfunction, increased aneuploidy and apoptotic cell death. In contrast, p53-proficient cells were able to tolerate HJURP depletion and prevent chromosomal instability by inducing cell cycle arrest. Finally, the authors showed that the dependence of p53 null tumor cells on HJURP for maintaining their hyperproliferative state could be exploited in a synthetic lethality strategy to induce tumor regression. A model based on the findings of Filipescu *et al*. (181) is presented in Figure 4A.

Recent experiments by Shresta *et al*. (8) have highlighted the contribution of mislocalized CENP-A to kinetochore defects and the CIN phenotype observed in many cancers, suggesting that ectopic distribution of CENP-A results in the depletion of essential centromere and kinetochore components, thereby weakening native kinetochores and leading to chromosomal instability. In their study, the mislocalization of CENP-A resulted in reduced centromeric levels of two key components involved in building the kinetochore-microtubule interface: CENP-T and Nuf2 (a component of the Ndc80 complex recruited by CENP-T) (76). Of note, in neither case was the decreased presence observed at the centromeres associated with a mislocalization to noncentromeric regions. Unlike CENP-T and Nuf2, however, CENP-A induction resulted in significant mislocalization of endogenous CENP-C, consistent with previous reports (9,40). This latter finding is in full agreement with the implication of CENP-A nucleosomes in the specific recruitment of CCAN members. Importantly, the CIN phenotype of CENP-A overexpressing cells could be rescued by depletion of DAXX, further stressing the importance of this chaperone in ectopic CENP-A assembly and expression of the CIN phenotype (8). A model depicting the impact of ectopic CENP-A on chromosome instability in cancer cells is presented in Figure 4B.

Although chromosomal instability is a driving force in tumorigenesis, excessive CIN (for instance, as seen upon depletion of HJURP in the p53-null cancer model system used by Filipescu *et al*. (181)) can cause unsustainable genotoxicity and/or mitotic stress and be deleterious (7). In this context, and since DAXX depletion prevents centromere/kinetochore dysregulation thus promoting chromosomal stability (8), it is notable that the expression levels of DAXX itself are found upregulated in a number of cancers (198–202). It is possible that the upregulation of DAXX is not sufficient to hinder the hyperproliferation phenotype of tumor cells. Alternatively, DAXX upregulation may correlate with alterations in safeguard mechanisms such that the increased genetic instability is tolerated. Finally, DAXX overexpression confers resistance to a variety of genotoxic agents (40) whereas its depletion sensitizes cells to UV light-and actinomycin-D induced DNA damage (203), suggesting that it may help cancer cells cope with the increased burden of endogenous DNA damage generated during DNA replication as a result of increased proliferation.

Finally, it is striking that partial loss of H3.3 in mouse through inactivation of H3f3b results in defective cell division and chromosome segregation, associated with increased pericentric heterochromatin and widespread ectopic CENP-A protein localization (204). Moreover, complete loss of H3.3 function (through targeting of both H3f3a and H3f3b) resulted in developmental retardation and in embryonic lethality during development (205). Examination of H3.3 knockout/p53*null* MEF lines revealed that loss of H3.3 caused increased mitotic abnormalities including lagging chromosomes and anaphase bridges resulting from dicentric chromosomes formed through telomeric fusions, with mitotic spreads displaying increased polyploidy and aneuploidy (205). Interestingly, loss of H3.3 caused dysfunction of heterochromatin structures, includ-

Figure 4. CENP-A deregulation and cancer. (**A**) Loss of p53 and oncogenic transformation lead to essential roles for CENP-A and its chaperone HJURP. In the model proposed by Filipescu *et al.* (181), the observed upregulation of CENP-A and HJURP in p53-null cells undergoing during malignant transformation allows cancer cells to sustain high proliferation rates by increasing the efficiency of CENP-A deposition and centromere propagation. Depletion of HJURP in such a model results in the rapid loss of CENP-A from the centromeres which, in cells lacking the cell-cycle arrest functions of p53, results in centromere dysfunction, chromosomal instability, aneuploidy and cell death. (**B**) Impact of CENP-A overexpression and ectopic localization on centromere dynamics and chromosome instability in cancer cells. Under normal conditions (upper panel), kinetochores assemble and function normally, providing sustained spindle microtubule attachment and enabling the orderly separation of sister chromatids. In CENP-A overexpressing cancer cells (lower panel), Shrestha *et al.* (8) propose that ectopic CENP-A molecules may titrate out and/or induce the loss of factors essential for proper kinetochore assembly and function at native centromeres, leading to chromosome segregation defects that are depicted here by aneuploidy and micronuclei formation.

ing at telomeres, pericentric regions and centromeres where increased CENP-A binding and H3K4me2 levels were observed (205). Recently, recurrent driving mutations affecting H3.3 (K27M, G34R/V and K36M) and its chaperones DAXX and ATRX have been reported in specific types of gliomas, soft tissue tumors and pancreatic neuroendocrine tumors (206–209). In addition to directly modifying residues at, or near, critical epigenetically regulated sites, it has been proposed that H3.3 mutations could alter recognition of H3.3 by chromatin remodeling enzymes or chaperones such as DAXX/ATRX, leading to aberrant mutant H3.3 incorporation and impacting chromosomal stability as observed upon H3.3 loss (210,211). It is also possible

that these mutations affect H3.3 turnover and induce conformational changes that disrupt normal chromatin architecture (210). In this regard, whether the mutations identified in H3.3 affect its proposed role as a placeholder for CENP-A during cell division remains to be investigated. Notably, H3F3A G34 mutations in pediatric glioblastoma caused widespread changes in H3K9me3 and H3K36me3 by interfering with the KDM4 family of K9/K36 demethylases (211) and were also associated with global DNA hypomethylation, which was particularly prominent in telomeric regions (212). In addition, the mutations affecting DAXX or ATRX in pancreatic neuroendocrine tumors and pediatric GBM were found to correlate with the loss of their

protein expression and an abnormal mechanism of telomere maintenance called Alternative Lengthening of Telomeres (ALT) (208,213,214), which is linked to chromosomal instability (215). Since DAXX-mediated ectopic deposition of CENP-A can occur at telomeres, future research will need to determine whether such a mislocalization can impact chromosomal stability through disruption of telomere function.

CONCLUSIONS

The epigenetic engineering experiments described in this review have had a major impact on our current knowledge of the mechanisms that underlie CENP-A deposition. They have identified the chromatin states and factors that facilitate/inhibit this process, and underlined the importance of some of the histone modification marks and other epigenetic features (such as transcription of the underlying DNA and the importance of the flanking heterochromatin) that contribute to the centrochromatin signature. As the tethering of chromatin and centromeric factors does not always allow the capture of subtle or ephemeral chromatin changes, future mechanistic insights will depend on continuous efforts to tackle the dynamics of centrochromatin and define the time windows during which the various chromatin factors operate. It will also be illuminating to learn the relative contribution of the diverse histone modifiers that catalyse each histone tail modification and in particular address the role of modifications (such as H3K9me2 and H3K27me2) that have hitherto been overlooked.

The elucidation of the distinct structure of CENP-A nucleosomes has greatly improved our understanding of CENP-A function. Likewise, the recent realization that CENP-A/H3.3 heterotypic nucleosomes can be assembled during the ectopic deposition of CENP-A in cancer cells opens new horizons in the investigation of the molecular mechanisms that govern ectopic CENP-A assembly and the epigenetic and pathological consequences of CENP-A mislocalization. Although common themes were found between the assembly of CENP-A at centromeres and its ectopic deposition, the available evidence, illustrated for instance by the interplay between DAXX and CENP-A and the presence of heterotypic nucleosome particles in cancer cells, suggests that the impact of CENP-A accumulation at non-centromeric sites also results from interactions that are not seen at native centromeres. In this regard, the recent discovery of recurrent driving mutations affecting H3.3 and its chaperones in specific types of gliomas and soft tissue tumors, and the realization that such mutations lead to a reformatting of transcriptional programs which is associated with tumorigenesis (209), raise the issue of whether CENP-A (and DAXX) deregulation and the ensuing mislocalization of CENP-A might also drive tumorigenesis in some cancer types by impacting the transcriptome. Finally, although genetic instability in CENP-A overexpressing cells results in part from a deregulation of the balance between CENP-A and its bona fide chaperone or the mobilization of essential centromere and kinetochore components away from native centromere, the current evidence also indicates that CENP-A overexpression confers resistance to DNA damage. Possible scenarios exploiting these features to kill cancer cells include strategies targeting the factors that protect against the lethal effects of CIN to which CENP-A overexpressing cells evolve an addiction, as well as strategies targeting CENP-A to sensitize cancer cells to genotoxic chemotherapeutics. In a nutshell, understanding the missregulations and promiscuous interactions that distort the native CENP-A biology carries important prospects in the fight against cancer.

REFERENCES

1. Earnshaw,W.C. and Rothfield,N. (1985) Identification of a family of human centromere proteins using autoimmune sera from patients with scleroderma. *Chromosoma*, **91**, 313–321.
2. Palmer,D.K., O'Day,K., Wener,M.H., Andrews,B.S. and Margolis,R.L. (1987) A 17-kD centromere protein (CENP-A) copurifies with nucleosome core particles and with histones. *J. Cell Biol.*, **104**, 805–815.
3. Palmer,D.K., O'Day,K., Trong,H.L., Charbonneau,H. and Margolis,R.L. (1991) Purification of the centromere-specific protein CENP-A and demonstration that it is a distinctive histone. *Proc. Natl. Acad. Sci. U.S.A.*, **88**, 3734–3738.
4. Sullivan,K.F., Hechenberger,M. and Masri,K. (1994) Human CENP-A contains a histone H3 related histone fold domain that is required for targeting to the centromere. *J. Cell Biol.*, **127**, 581–592.
5. Regnier,V., Vagnarelli,P., Fukagawa,T., Zerjal,T., Burns,E., Trouche,D., Earnshaw,W. and Brown,W. (2005) CENP-A is required for accurate chromosome segregation and sustained kinetochore association of BubR1. *Mol. Cell. Biol.*, **25**, 3967–3981.
6. Heun,P., Erhardt,S., Blower,M.D., Weiss,S., Skora,A.D. and Karpen,G.H. (2006) Mislocalization of the drosophila centromere-specific histone CID promotes formation of functional ectopic kinetochores. *Dev. Cell*, **10**, 303–315.
7. Zhang,W., Mao,J.H., Zhu,W., Jain,A.K., Liu,K., Brown,J.B. and Karpen,G.H. (2016) Centromere and kinetochore gene misexpression predicts cancer patient survival and response to radiotherapy and chemotherapy. *Nat. Commun.*, **7**, 12619.
8. Shrestha,R.L., Ahn,G.S., Staples,M.I., Sathyan,K.M., Karpova,T.S., Foltz,D.R. and Basrai,M.A. (2017) Mislocalization of centromeric histone H3 variant CENP-A contributes to chromosomal instability (CIN) in human cells. *Oncotarget*, **8**, 46781–46800.
9. Hooser,A.A.Van., Ouspenski,I.I., Gregson,H.C., Starr,D.A., Yen,T.J., Goldberg,M.L., Yokomori,K., Earnshaw,W.C., Sullivan,K.F. and Brinkley,B.R. (2001) Specification of kinetochore-forming chromatin by the histone H3 variant CENP-A. *J. Cell Sci.*, **114**, 3529–3542.

10. Olszak,A.M., van Essen,D., Pereira,A.J., Diehl,S., Manke,T., Maiato,H., Saccani,S. and Heun,P. (2011) Heterochromatin boundaries are hotspots for de novo kinetochore formation. *Nat. Cell Biol.*, **13**, 799–808.

11. Gonzalez,M., He,H., Dong,Q., Sun,S. and Li,F. (2014) Ectopic centromere nucleation by CENP-A in fission yeast. *Genetics*, **198**, 1433–1446.

12. Athwal,R.K., Walkiewicz,M.P., Baek,S., Fu,S., Bui,M., Camps,J., Ried,T., Sung,M.-H. and Dalal,Y. (2015) CENP-A nucleosomes localize to transcription factor hotspots and subtelomeric sites in human cancer cells. *Epigenet. Chromatin*, **8**, 2.

13. Sun,X., Clermont,P.L., Jiao,W., Helgason,C.D., Gout,P.W., Wang,Y. and Qu,S. (2016) Elevated expression of the centromere protein-A(CENP-A)-encoding gene as a prognostic and predictive biomarker in human cancers. *Int. J. Cancer*, **139**, 899–907.

14. McGovern,S.L., Qi,Y., Pusztai,L., Symmans,W.F. and Buchholz,Ta. (2012) Centromere protein-A, an essential centromere protein, is a prognostic marker for relapse in estrogen receptor-positive breast cancer. *Breast Cancer Res.*, **14**, R72.

15. Yuen,K.W.Y., Montpetit,B. and Hieter,P. (2005) The kinetochore and cancer: What's the connection? *Curr. Opin. Cell Biol.*, **17**, 576–582.

16. Stangeland,B., Mughal,A.A., Grieg,Z., Sandberg,C.J., Joel,M., Nygård,S., Meling,T., Murrell,W., Mo,E.O.V. and Langmoen,I.A. (2015) Combined expressional analysis, bioinformatics and targeted proteomics identify new potential therapeutic targets in glioblastoma stem cells. *Oncotarget*, **6**, 26192–26215.

17. Sekulic,N., Bassett,E.A., Rogers,D.J. and Black,B.E. (2010) The structure of (CENP-A/H4)2 reveals physical features that mark centromeres. *Structure*, **467**, 347–351.

18. Tachiwana,H., Kagawa,W., Shiga,T., Osakabe,A., Miya,Y., Saito,K., Hayashi-Takanaka,Y., Oda,T., Sato,M., Park,S.-Y. *et al.* (2011) Crystal structure of the human centromeric nucleosome containing CENP-A. *Nature*, **476**, 232–235.

19. Kato,H., Jiang,J., Zhou,B.-R., Rozendaal,M., Feng,H., Ghirlando,R., Xiao,T.S., Straight,A.F. and Bai,Y. (2013) A conserved mechanism for centromeric nucleosome recognition by centromere protein CENP-C. *Science*, **340**, 1110–1113.

20. van Holde,K.E. (1989) Chromatin. *Cell*, **59**, 243–244.

21. Palmer,D.K., O'Day,K. and Margolis,R. (1989) Biochemical analysis of CENP-A, a centromeric protein with histone-like properties. *Prog. Clin. Biol. Res.*, **318**, 61–72.

22. Talbert,P.B. and Henikoff,S. (2010) Histone variants–ancient wrap artists of the epigenome. *Nat Rev Mol Cell Biol*, **11**, 264–275.

23. Strahl,B.D. and Allis,C.D. (2000) The language of covalent histone modifications. *Nature*, **403**, 41–45.

24. Boulard,M., Bouvet,P., Kundu,T.K. and Dimitrov,S. (2007) Histone variant nucleosomes: structure, function and implication in disease. *Subcell. Biochem.*, **41**, 71–89.

25. Gatchalian,J., Wang,X., Ikebe,J., Cox,K.L., Tencer,A.H., Zhang,Y., Burge,N.L., Di,L., Gibson,M.D., Musselman,C.A. *et al.* (2017) Accessibility of the histone H3 tail in the nucleosome for binding of paired readers. *Nat. Commun.*, **8**, 1489.

26. Bailey,A.O., Panchenko,T., Sathyan,K.M., Petkowski,J.J., Pai,P.-J., Bai,D.L., Russell,D.H., Macara,I.G., Shabanowitz,J., Hunt,D.F. *et al.* (2013) Posttranslational modification of CENP-A influences the conformation of centromeric chromatin. *Proc. Natl. Acad. Sci. U.S.A.*, **110**, 11827–11832.

27. Srivastava,S., Zasadzińska,E. and Foltz,D.R. (2018) Posttranslational mechanisms controlling centromere function and assembly. *Curr. Opin. Cell Biol.*, **52**, 126–135.

28. Goutte-Gattat,D., Shuaib,M., Ouararhni,K., Gautier,T., Skoufias,D.A., Hamiche,A. and Dimitrov,S. (2013) Phosphorylation of the CENP-A amino-terminus in mitotic centromeric chromatin is required for kinetochore function. *Proc. Natl. Acad. Sci. U.S.A.*, **110**, 8579–8584.

29. Zeitlin,S.G., Barber,C.M., Allis,C.D. and Sullivan,K. (2001) Differential regulation of CENP-A and histone H3 phosphorylation in G2/M. *J. Cell Sci.*, **114**, 653–661.

30. Tachiwana,H., Kagawa,W. and Kurumizaka,H. (2012) Comparison between the CENP-A and histone H3 structures in nucleosomes. *Nucleus*, **3**, 6–11.

31. Nechemia-Arbely,Y., Fachinetti,D., Miga,K.H., Sekulic,N., Soni,G.V., Kim,D.H., Wong,A.K., Lee,A.Y., Nguyen,K., Dekker,C.

et al. (2017) Human centromeric CENP-A chromatin is a homotypic, octameric nucleosome at all cell cycle points. *J. Cell Biol.*, **216**, 607–621.

32. Cheema,M.S. and Ausió,J. (2015) The structural determinants behind the epigenetic role of histone variants. *Genes (Basel).*, **6**, 685–713.

33. Pentakota,S., Zhou,K., Smith,C., Maffini,S., Petrovic,A., Morgan,G.P., Weir,J.R., Vetter,I.R., Musacchio,A. and Luger,K. (2017) Decoding the centromeric nucleosome through CENP-N. *Elife*, **6**, e33442.

34. Luger,K., Mäder,A.W., Richmond,R.K., Sargent,D.F. and Richmond,T.J. (1997) Crystal structure of the nucleosome core particle at 2.8 A resolution. *Nature*, **389**, 251–260.

35. Roulland,Y., Ouararhni,K., Naidenov,M., Ramos,L., Shuaib,M., Syed,S.H., Lone,I.N., Boopathi,R., Fontaine,E., Papai,G. *et al.* (2016) The Flexible Ends of CENP-A Nucleosome Are Required for Mitotic Fidelity. *Mol. Cell*, **63**, 674–685.

36. Cole,H.A., Howard,B.H. and Clark,D.J. (2011) The centromeric nucleosome of budding yeast is perfectly positioned and covers the entire centromere. *Proc. Natl. Acad. Sci. U.S.A.*, **108**, 12687–12692.

37. Bednar,J., Garcia-Saez,I., Boopathi,R., Cutter,A.R., Papai,G., Reymer,A., Syed,S.H., Lone,I.N., Tonchev,O., Crucifix,C. *et al.* (2017) Structure and dynamics of a 197 bp nucleosome in complex with linker histone H1. *Mol. Cell*, **66**, 384–397.

38. Bayona-Feliu,A., Casas-Lamesa,A., Reina,O., Bernués,J. and Azorín,F. (2017) Linker histone H1 prevents R-loop accumulation and genome instability in heterochromatin. *Nat. Commun.*, **8**, 283.

39. Moreno-Moreno,O., Torras-Llort,M. and Azorín,F. (2017) Variations on a nucleosome theme: the structural basis of centromere function. *Bioessays*, **39**, 1600241.

40. Lacoste,N., Woolfe,A., Tachiwana,H., Garea,A., Barth,T., Cantaloube,S., Kurumizaka,H., Imhof,A. and Almouzni,G. (2014) Mislocalization of the centromeric histone variant CenH3/CENP-A in human cells depends on the chaperone DAXX. *Mol. Cell*, **53**, 631–644.

41. Arimura,Y., Shirayama,K., Horikoshi,N., Fujita,R., Taguchi,H., Kagawa,W., Fukagawa,T., Almouzni,G. and Kurumizaka,H. (2015) Crystal structure and stable property of the cancer-associated heterotypic nucleosome containing CENP-A and H3.3. *Sci. Rep.*, **4**, 7115.

42. Hake,S.B. and Allis,C.D. (2006) Histone H3 variants and their potential role in indexing mammalian genomes: the 'H3 barcode hypothesis'. *Proc. Natl. Acad. Sci. U.S.A.*, **103**, 6428–6435.

43. Henikoff,S. and Henikoff,J.G. (2012) 'Point' centromeres of saccharomyces harbor single centromere-specific nucleosomes. *Genetics*, **190**, 1575–1577.

44. Biggins,S. (2013) The composition, functions, and regulation of the budding yeast kinetochore. *Genetics*, **194**, 817–846.

45. Ohkuni,K. and Kitagawa,K. (2011) Endogenous transcription at the centromere facilitates centromere activity in budding yeast. *Curr. Biol.*, **21**, 1695–1703.

46. Baker,R.E. and Masison,D.C. (1990) Isolation of the gene encoding the Saccharomyces cerevisiae centromere-binding protein CP1. *Mol. Cell. Biol.*, **10**, 2458–2467.

47. Cai,M. and Davis,R.W. (1990) Yeast centromere binding protein CBF1, of the helix-loop-helix protein family, is required for chromosome stability and methionine prototrophy. *Cell*, **61**, 437–446.

48. Henikoff,S., Ramachandran,S., Krassovsky,K., Bryson,T.D., Codomo,C.A., Brogaard,K., Widom,J., Wang,J.-P. and Henikoff,J.G. (2014) The budding yeast Centromere DNA Element II wraps a stable Cse4 hemisome in either orientation in vivo. *Elife*, **3**, e01861.

49. Jehn,B., Niedenthal,R. and Hegemann,J.H. (1991) In vivo analysis of the Saccharomyces cerevisiae centromere CDEIII sequence: requirements for mitotic chromosome segregation. *Mol. Cell. Biol.*, **11**, 5212–5221.

50. Lechner,J. and Carbon,J. (1991) A 240 kd multisubunit protein complex, CBF3, is a major component of the budding yeast centromere. *Cell*, **64**, 717–725.

51. Buscaino,A., Allshire,R. and Pidoux,A. (2010) Building centromeres: home sweet home or a nomadic existence? *Curr. Opin. Genet. Dev.*, **20**, 118–126.

52. Fukagawa,T. and Earnshaw,W.C. (2014) The centromere: chromatin foundation for the kinetochore machinery. *Dev. Cell*, **30**, 496–508.
53. Pidoux,A.L. and Allshire,R.C. (2005) The role of heterochromatin in centromere function. *Philos. Trans. R. Soc. B Biol. Sci.*, **360**, 569–579.
54. Willard,H.F. (1985) Chromosome-specific organization of human alpha satellite DNA. *Am. J. Hum. Genet.*, **37**, 524–532.
55. Willard,H.F. and Waye,J.S. (1987) Hierarchical order in chromosome-specific human alpha satellite DNA. *Trends Genet.*, **3**, 192–198.
56. Castillo,A.G., Mellone,B.G., Partridge,J.F., Richardson,W., Hamilton,G.L., Allshire,R.C. and Pidoux,A.L. (2007) Plasticity of fission Yeast CENP-A chromatin driven by relative levels of histone H3 and H4. *PLoS Genet.*, **3**, e121.
57. Ho,K., Tsuchiya,D., Oliger,A.C. and Lacefield,S. (2014) Localization and function of budding yeast CENP-A depends upon kinetochore protein interactions and is independent of canonical centromere sequence. *Cell Rep.*, **9**, 2027–2033.
58. Kobayashi,N., Suzuki,Y., Schoenfeld,L.W., Müller,C.A., Nieduszynski,C., Wolfe,K.H. and Tanaka,T.U. (2015) Discovery of an unconventional centromere in budding yeast redefines evolution of point centromeres. *Curr. Biol.*, **25**, 2026–2033.
59. Chatterjee,G., Sankaranarayanan,S.R., Guin,K., Thattikota,Y., Padmanabhan,S., Siddharthan,R. and Sanyal,K. (2016) Repeat-Associated fission Yeast-Like regional centromeres in the ascomycetous budding yeast candida tropicalis. *PLOS Genet.*, **12**, e1005839.
60. Montefalcone,G. (1999) Centromere repositioning. *Genome Res.*, **9**, 1184–1188.
61. Piras,F.M., Nergadze,S.G., Magnani,E., Bertoni,L., Attolini,C., Khoriauli,L., Raimondi,E. and Giulotto,E. (2010) Uncoupling of Satellite DNA and centromeric function in the genus equus. *PLoS Genet.*, **6**, e1000845.
62. Locke,D.P., Hillier,L.W., Warren,W.C., Worley,K.C., Nazareth,L.V., Muzny,D.M., Yang,S., Wang,Z., Chinwalla,A.T., Minx,P. *et al.* (2011) Comparative and demographic analysis of orang-utan genomes. *Nature*, **469**, 529–533.
63. Allshire,R.C. and Karpen,G.H. (2008) Epigenetic regulation of centromeric chromatin: old dogs, new tricks? *Nat. Rev. Genet.*, **9**, 923–937.
64. Black,B.E. and Cleveland,D.W. (2011) Epigenetic centromere propagation and the nature of CENP-a nucleosomes. *Cell*, **144**, 471–479.
65. Fachinetti,D., Folco,H.D., Nechemia-Arbely,Y., Valente,L.P., Nguyen,K., Wong,A.J., Zhu,Q., Holland,A.J., Desai,A., Jansen,L.E.T. *et al.* (2013) A two-step mechanism for epigenetic specification of centromere identity and function. *Nat. Cell Biol.*, **15**, 1056–1066.
66. Sullivan,Ba. and Karpen,G.H. (2004) Centromeric chromatin exhibits a histone modification pattern that is distinct from both euchromatin and heterochromatin. *Nat. Struct. Mol. Biol.*, **11**, 1076–1083.
67. Ross,J.E., Woodlief,K.S. and Sullivan,B.A. (2016) Inheritance of the CENP-A chromatin domain is spatially and temporally constrained at human centromeres. *Epigenet. Chromatin*, **9**, 20.
68. Bergmann,J.H., Rodríguez,M.G., Martins,N.M.C., Kimura,H., Kelly,Da., Masumoto,H., Larionov,V., Jansen,L.E.T. and Earnshaw,W.C. (2011) Epigenetic engineering shows H3K4me2 is required for HJURP targeting and CENP-A assembly on a synthetic human kinetochore. *EMBO J.*, **30**, 328–340.
69. Lam,A.L., Boivin,C.D., Bonney,C.F., Rudd,M.K. and Sullivan,B.A. (2006) Human centromeric chromatin is a dynamic chromosomal domain that can spread over noncentromeric DNA. *Proc. Natl. Acad. Sci. U. S. A.*, **103**, 4186–4191.
70. Black,B.E. and Cleveland,D.W. (2011) Epigenetic centromere propagation and the nature of CENP-a nucleosomes. *Cell*, **144**, 471–479.
71. Ohzeki,J., Bergmann,J.H., Kouprina,N., Noskov,V.N., Nakano,M., Kimura,H., Earnshaw,W.C., Larionov,V. and Masumoto,H. (2012) Breaking the HAC Barrier: histone H3K9 acetyl/methyl balance regulates CENP-A assembly. *EMBO J.*, **31**, 2391–2402.
72. Ribeiro,S.A., Vagnarelli,P., Dong,Y., Hori,T., McEwen,B.F., Fukagawa,T., Flors,C. and Earnshaw,W.C. (2010) A
73. Bailey,A.O., Panchenko,T., Shabanowitz,J., Lehman,S.M., Bai,D.L., Hunt,D.F., Black,B.E. and Foltz,D.R. (2016) Identification of the Post-translational modifications present in centromeric chromatin. *Mol. Cell. Proteomics*, **15**, 918–931.
74. Hori,T., Shang,W.-H., Toyoda,A., Misu,S., Monma,N., Ikeo,K., Molina,O., Vargiu,G., Fujiyama,A., Kimura,H. *et al.* (2014) Histone H4 Lys 20 monomethylation of the CENP-A nucleosome is essential for kinetochore assembly. *Dev. Cell*, **29**, 740–749.
75. Martins,N.M.C., Bergmann,J.H., Shono,N., Kimura,H., Larionov,V., Masumoto,H. and Earnshaw,W.C. (2016) Epigenetic engineering shows that a human centromere resists silencing mediated by H3K27me3/K9me3. *Mol. Biol. Cell*, **27**, 177–196.
76. Hinshaw,S.M. and Harrison,S.C. (2018) Kinetochore function from the bottom up. *Trends Cell Biol.*, **28**, 22–33.
77. Perpelescu,M. and Fukagawa,T. (2011) The ABCs of CENPs. *Chromosoma*, **120**, 425–446.
78. Barth,T.K., Schade,G.O.M., Schmidt,A., Vetter,I., Wirth,M., Heun,P., Thomae,A.W. and Imhof,A. (2014) Identification of novel Drosophila centromere-associated proteins. *Proteomics*, **14**, 2167–2178.
79. Earnshaw,W.C. (2015) Discovering centromere proteins: from cold white hands to the A, B, C of CENPs. *Nat. Rev. Mol. Cell Biol.*, **16**, 443–449.
80. Foltz,D.R., Jansen,L.E.T., Bailey,A.O., Yates,J.R., Bassett,E.A., Wood,S., Black,B.E. and Cleveland,D.W. (2009) Centromere-specific assembly of CENP-a nucleosomes is mediated by HJURP. *Cell*, **137**, 472–484.
81. Dunleavy,E.M., Roche,D., Tagami,H., Lacoste,N., Ray-Gallet,D., Nakamura,Y., Daigo,Y., Nakatani,Y. and Almouzni-Pettinotti,G. (2009) HJURP is a cell-cycle-dependent maintenance and deposition factor of CENP-A at centromeres. *Cell*, **137**, 485–497.
82. Shuaib,M., Ouararhni,K., Dimitrov,S. and Hamiche,A. (2010) HJURP binds CENP-A via a highly conserved N-terminal domain and mediates its deposition at centromeres. *Proc. Natl. Acad. Sci. U.S.A.*, **107**, 1349–1354.
83. Moree,B., Meyer,C.B., Fuller,C.J. and Straight,A.F. (2011) CENP-C recruits M18BP1 to centromeres to promote CENP-A chromatin assembly. *J. Cell Biol.*, **194**, 855–871.
84. Dambacher,S., Deng,W., Hahn,M., Sadic,D., Fröhlich,J., Nuber,A., Hoischen,C., Diekmann,S., Leonhardt,H. and Schotta,G. (2012) CENP-C facilitates the recruitment of M18BP1 to centromeric chromatin. *Nucleus*, **3**, 101–110.
85. Wang,J., Liu,X., Dou,Z., Chen,L., Jiang,H., Fu,C., Fu,G., Liu,D., Zhang,J., Zhu,T. *et al.* (2014) Mitotic regulator Mis18β interacts with and specifies the centromeric assembly of molecular chaperone holliday junction recognition protein (HJURP). *J. Biol. Chem.*, **289**, 8326–8336.
86. Silva,M.C.C., Bodor,D.L., Stellfox,M.E., Martins,N.M.C., Hochegger,H., Foltz,D.R. and Jansen,L.E.T. (2012) Cdk activity couples epigenetic centromere inheritance to cell cycle progression. *Dev. Cell*, **22**, 52–63.
87. McKinley,K.L. and Cheeseman,I.M. (2014) Polo-like kinase 1 licenses CENP-A deposition at centromeres. *Cell*, **158**, 397–411.
88. Spiller,F., Medina-Pritchard,B., Abad,M.A., Wear,M.A., Molina,O., Earnshaw,W.C. and Jeyaprakash,A.A. (2017) Molecular basis for Cdk1-regulated timing of Mis18 complex assembly and CENP-A deposition. *EMBO Rep.*, **18**, 894–905.
89. Shono,N., Ohzeki,J.-I., Otake,K., Martins,N.M.C., Nagase,T., Kimura,H., Larionov,V., Earnshaw,W.C. and Masumoto,H. (2015) CENP-C and CENP-I are key connecting factors for kinetochore and CENP-A assembly. *J. Cell Sci.*, **128**, 4572–4587.
90. Perpelescu,M., Nozaki,N., Obuse,C., Yang,H. and Yoda,K. (2009) Active establishment of centromeric CENP-A chromatin by RSF complex. *J. Cell Biol.*, **185**, 397–407.
91. Lagana,A., Dorn,J.F., De Rop,V., Ladouceur,A.-M., Maddox,A.S. and Maddox,P.S. (2010) A small GTPase molecular switch regulates epigenetic centromere maintenance by stabilizing newly incorporated CENP-A. *Nat. Cell Biol.*, **12**, 1186–1193.
92. Schuh,M., Lehner,C.F. and Heidmann,S. (2007) Incorporation of Drosophila CID/CENP-A and CENP-C into centromeres during early embryonic anaphase. *Curr. Biol.*, **17**, 237–243.

93. Schittenhelm,R.B., Althoff,F., Heidmann,S. and Lehner,C.F. (2010) Detrimental incorporation of excess Cenp-A/Cid and Cenp-C into Drosophila centromeres is prevented by limiting amounts of the bridging factor Cal1. *J. Cell Sci.*, **123**, 3768–3779.

94. Bade,D., Pauleau,A.-L., Wendler,A. and Erhardt,S. (2014) The E3 ligase CUL3/RDX controls centromere maintenance by ubiquitylating and stabilizing CENP-A in a CAL1-dependent manner. *Dev. Cell*, **28**, 508–519.

95. Chen,C.-C., Dechassa,M.L., Bettini,E., Ledoux,M.B., Belisario,C., Heun,P., Luger,K. and Mellone,B.G. (2014) CAL1 is the Drosophila CENP-A assembly factor. *J. Cell Biol.*, **204**, 313–329.

96. Barnhart,M.C., Kuich,P.H.J.L., Stellfox,M.E., Ward,J.A., Bassett,E.A., Black,B.E. and Foltz,D.R. (2011) HJURP is a CENP-A chromatin assembly factor sufficient to form a functional de novo kinetochore. *J. Cell Biol.*, **194**, 229–243.

97. Dunleavy,E.M., Almouzni,G. and Karpen,G.H. (2011) H3.3 is deposited at centromeres in S phase as a placeholder for newly assembled CENP-A in G^1 phase. *Nucleus*, **2**, 146–157.

98. Stellfox,M.E., Bailey,A.O. and Foltz,D.R. (2013) Putting CENP-A in its place. *Cell. Mol. Life Sci.*, **70**, 387–406.

99. Wong,L.H., Brettingham-Moore,K.H., Chan,L., Quach,J.M., Anderson,M.A., Northrop,E.L., Hannan,R., Saffery,R., Shaw,M.L., Williams,E. *et al.* (2007) Centromere RNA is a key component for the assembly of nucleoproteins at the nucleolus and centromere. *Genome Res.*, **17**, 1146–1160.

100. Ideue,T., Cho,Y., Nishimura,K. and Tani,T. (2014) Involvement of satellite I noncoding RNA in regulation of chromosome segregation. *Genes Cells*, **19**, 528–538.

101. Blower,M.D. (2016) Centromeric transcription regulates Aurora-B localization and activation. *Cell Rep.*, **15**, 1624–1633.

102. Chan,F.L., Marshall,O.J., Saffery,R., Kim,B.W., Earle,E., Choo,K.H.A. and Wong,L.H. (2012) Active transcription and essential role of RNA polymerase II at the centromere during mitosis. *Proc. Natl. Acad. Sci. U.S.A.*, **109**, 1979–1984.

103. McNulty,S.M., Sullivan,L.L. and Sullivan,B.A. (2017) Human centromeres produce chromosome-specific and array-specific alpha satellite transcripts that are complexed with CENP-A and CENP-C. *Dev. Cell*, **42**, 226–240.

104. Bobkov,G.O.M., Gilbert,N. and Heun,P. (2018) Centromere transcription allows CENP-A to transit from chromatin association to stable incorporation. *J. Cell Biol.*, **217**, 1957–1972.

105. Liu,H., Qu,Q., Warrington,R., Rice,A., Cheng,N. and Yu,H. (2015) Mitotic transcription installs Sgo1 at centromeres to coordinate chromosome segregation. *Mol. Cell*, **59**, 426–436.

106. Quénet,D. and Dalal,Y. (2014) A long non-coding RNA is required for targeting centromeric protein A to the human centromere. *Elife*, **3**, e03254.

107. Talbert,P.B. and Henikoff,S. (2018) Transcribing centromeres: noncoding RNAs and kinetochore assembly. *Trends Genet.*, **34**, 587–599.

108. Chen,C.-C., Bowers,S., Lipinszki,Z., Palladino,J., Trusiak,S., Bettini,E., Rosin,L., Przewloka,M.R., Glover,D.M., O'Neill,R.J. *et al.* (2015) Establishment of centromeric chromatin by the CENP-A assembly factor CAL1 requires FACT-mediated transcription. *Dev. Cell*, **34**, 73–84.

109. Maloney,K.A., Sullivan,L.L., Matheny,J.E., Strome,E.D., Merrett,S.L., Ferris,A. and Sullivan,B.A. (2012) Functional epialleles at an endogenous human centromere. *Proc. Natl. Acad. Sci. U.S.A.*, **109**, 13704–13709.

110. Okada,T., Ohzeki,J., Nakano,M., Yoda,K., Brinkley,W.R., Larionov,V. and Masumoto,H. (2007) CENP-B controls centromere formation depending on the chromatin context. *Cell*, **131**, 1287–1300.

111. Shandilya,J., Senapati,P., Hans,F., Menoni,H., Bouvet,P., Dimitrov,S., Angelov,D. and Kundu,T.K. (2014) Centromeric histone variant CENP-A represses acetylation-dependent chromatin transcription that is relieved by histone chaperone NPM1. *J. Biochem.*, **156**, 221–227.

112. Molina,O., Vargiu,G., Abad,M.A., Zhiteneva,A., Jeyaprakash,A.A., Masumoto,H., Kouprina,N., Larionov,V. and Earnshaw,W.C. (2016) Epigenetic engineering reveals a balance between histone modifications and transcription in kinetochore maintenance. *Nat. Commun.*, **7**, 13334.

113. Rošić,S., Köhler,F. and Erhardt,S. (2014) Repetitive centromeric satellite RNA is essential for kinetochore formation and cell division. *J. Cell Biol.*, **207**, 335–349.

114. Ohno,M., Fukagawa,T., Lee,J.S. and Ikemura,T. (2002) Triplex-forming DNAs in the human interphase nucleus visualized in situ by polypurine/polypyrimidine DNA probes and antitriplex antibodies. *Chromosoma*, **111**, 201–213.

115. Jonstrup,A.T., Thomsen,T., Wang,Y., Knudsen,B.R., Koch,J. and Andersen,A.H. (2008) Hairpin structures formed by alpha satellite DNA of human centromeres are cleaved by human topoisomerase IIalpha. *Nucleic Acids Res.*, **36**, 6165–6174.

116. Garavís,M., Escaja,N., Gabelica,V., Villasante,A. and González,C. (2015) Centromeric Alpha-Satellite DNA adopts dimeric i-Motif structures capped by AT hoogsteen base pairs. *Chemistry*, **21**, 9816–9824.

117. Aze,A., Sannino,V., Soffientini,P., Bachi,A. and Costanzo,V. (2016) Centromeric DNA replication reconstitution reveals DNA loops and ATR checkpoint suppression. *Nat. Cell Biol.*, **18**, 684–691.

118. Garavís,M., Méndez-Lago,M., Gabelica,V., Whitehead,S.L., González,C. and Villasante,A. (2015) The structure of an endogenous Drosophila centromere reveals the prevalence of tandemly repeated sequences able to form i-motifs. *Sci. Rep.*, **5**, 13307.

119. Kasinathan,S. and Henikoff,S. (2018) Non-B-form DNA is enriched at centromeres. *Mol. Biol. Evol.*, **35**, 949–962.

120. Goldberg,I.G., Sawhney,H., Pluta,A.F., Warburton,P.E. and Earnshaw,W.C. (1996) Surprising deficiency of CENP-B binding sites in African green monkey alpha-satellite DNA: implications for CENP-B function at centromeres. *Mol. Cell. Biol.*, **16**, 5156–5168.

121. Kato,T., Sato,N., Hayama,S., Yamabuki,T., Ito,T., Miyamoto,M., Kondo,S., Nakamura,Y. and Daigo,Y. (2007) Activation of Holliday junction recognizing protein involved in the chromosomal stability and immortality of cancer cells. *Cancer Res.*, **67**, 8544–8553.

122. Tanaka,Y., Nureki,O., Kurumizaka,H., Fukai,S., Kawaguchi,S., Ikuta,M., Iwahara,J., Okazaki,T. and Yokoyama,S. (2001) Crystal structure of the CENP-B protein-DNA complex: the DNA-binding domains of CENP-B induce kinks in the CENP-B box DNA. *EMBO J.*, **20**, 6612–6618.

123. Kabeche,L., Nguyen,H.D., Buisson,R. and Zou,L. (2018) A mitosis-specific and R loop-driven ATR pathway promotes faithful chromosome segregation. *Science*, **359**, 108–114.

124. Hewawasam,G., Shivaraju,M., Mattingly,M., Venkatesh,S., Martin-Brown,S., Florens,L., Workman,J.L. and Gerton,J.L. (2010) Psh1 is an E3 ubiquitin ligase that targets the centromeric histone variant Cse4. *Mol. Cell*, **40**, 444–454.

125. Canzonetta,C., Vernarecci,S., Iuliani,M., Marracino,C., Belloni,C., Ballario,P. and Filetici,P. (2015) SAGA DUB-Ubp8 deubiquitylates centromeric histone variant Cse4. *G3 (Bethesda)*, **6**, 287–298.

126. Ranjitkar,P., Press,M.O., Yi,X., Baker,R., MacCoss,M.J. and Biggins,S. (2010) An E3 ubiquitin ligase prevents ectopic localization of the centromeric histone H3 variant via the centromere targeting domain. *Mol. Cell*, **40**, 455–464.

127. Hewawasam,G.S., Mattingly,M., Venkatesh,S., Zhang,Y., Florens,L., Workman,J.L. and Gerton,J.L. (2014) Phosphorylation by casein kinase 2 facilitates Psh1 protein-assisted degradation of Cse4 protein. *J. Biol. Chem.*, **289**, 29297–29309.

128. Deyter,G.M.R., Hildebrand,E.M., Barber,A.D. and Biggins,S. (2017) Histone H4 facilitates the proteolysis of the budding yeast CENP-ACse4 centromeric histone variant. *Genetics*, **205**, 113–124.

129. Au,W.C., Dawson,A.R., Rawson,D.W., Taylor,S.B., Baker,R.E. and Basrai,M.A. (2013) A novel role of the N terminus of budding yeast histone H3 variant Cse4 in ubiquitin-mediated proteolysis. *Genetics*, **194**, 513–518.

130. Mishra,P.K., Guo,J., Dittman,L.E., Haase,J., Yeh,E., Bloom,K. and Basrai,M.A. (2015) Pat1 protects centromere-specific histone H3 variant Cse4 from Psh1-mediated ubiquitination. *Mol. Biol. Cell*, **26**, 2067–2079.

131. Ohkuni,K., Abdulle,R. and Kitagawa,K. (2014) Degradation of centromeric histone H3 variant Cse4 requires the Fpr3 peptidyl-prolyl Cis-Trans isomerase. *Genetics*, **196**, 1041–1045.

132. Cheng,H., Bao,X. and Rao,H. (2016) The F-box protein Rcy1 is involved in the degradation of histone H3 variant Cse4 and genome maintenance. *J. Biol. Chem.*, **291**, 10372–10377.

133. Ohkuni,K., Takahashi,Y., Fulp,A., Lawrimore,J., Au,W.-C., Pasupala,N., Levy-Myers,R., Warren,J., Strunnikov,A., Baker,R.E. *et al.* (2016) SUMO-Targeted Ubiquitin Ligase (STUbL) Slx5 regulates proteolysis of centromeric histone H3 variant Cse4 and prevents its mislocalization to euchromatin. *Mol. Biol. Cell*, **27**, 1500–1510.

134. Kitagawa,T., Ishii,K., Takeda,K. and Matsumoto,T. (2014) The 19S proteasome subunit Rpt3 regulates distribution of CENP-A by associating with centromeric chromatin. *Nat. Commun.*, **5**, 3597.

135. Moreno-Moreno,O., Torras-Llort,M. and Azorín,F. (2006) Proteolysis restricts localization of CID, the centromere-specific histone H3 variant of Drosophila, to centromeres. *Nucleic Acids Res.*, **34**, 6247–6255.

136. Moreno-Moreno,O., Medina-Giró,S., Torras-Llort,M. and Azorín,F. (2011) The F box protein partner of paired regulates stability of Drosophila centromeric histone H3, CenH3(CID). *Curr. Biol.*, **21**, 1488–1493.

137. Lomonte,P., Sullivan,K.F. and Everett,R.D. (2001) Degradation of nucleosome-associated centromeric histone H3-like protein CENP-A induced by herpes simplex virus type 1 protein ICP0. *J. Biol. Chem.*, **276**, 5829–5835.

138. Mendiburo,M.J., Padeken,J., Fülöp,S., Schepers,A. and Heun,P. (2011) Drosophila CENH3 is sufficient for centromere formation. *Science*, **334**, 686–690.

139. Nakano,M., Cardinale,S., Noskov,V.N., Gassmann,R., Vagnarelli,P., Kandels-Lewis,S., Larionov,V., Earnshaw,W.C. and Masumoto,H. (2008) Inactivation of a human kinetochore by specific targeting of chromatin modifiers. *Dev. Cell*, **14**, 507–522.

140. Molina,O., Kouprina,N., Masumoto,H., Larionov,V. and Earnshaw,W.C. (2017) Using human artificial chromosomes to study centromere assembly and function. *Chromosoma*, **126**, 559–575.

141. Folco,H.D., Pidoux,A.L., Urano,T. and Allshire,R.C. (2008) Heterochromatin and RNAi are required to establish CENP-A chromatin at centromeres. *Science*, **319**, 94–97.

142. Kagansky,A., Folco,H.D., Almeida,R., Pidoux,A.L., Boukaba,A., Simmer,F., Urano,T., Hamilton,G.L. and Allshire,R.C. (2009) Synthetic heterochromatin bypasses RNAi and centromeric repeats to establish functional centromeres. *Science*, **324**, 1716–1719.

143. Yang,J., Sun,S., Zhang,S., Gonzalez,M., Dong,Q., Chi,Z., Chen,Y.-H. and Li,F. (2018) Heterochromatin and RNAi regulate centromeres by protecting CENP-A from ubiquitin-mediated degradation. *PLoS Genet.*, **14**, e1007572.

144. Lehnertz,B., Ueda,Y., Derijck,A.A.H.A., Braunschweig,U., Perez-Burgos,L., Kubicek,S., Chen,T., Li,E., Jenuwein,T. and Peters,A.H.F.M. (2003) Suv39h-mediated histone H3 lysine 9 methylation directs DNA methylation to major satellite repeats at pericentric heterochromatin. *Curr. Biol.*, **13**, 1192–1200.

145. Loyola,A., Tagami,H., Bonaldi,T., Roche,D., Quivy,J.P., Imhof,A., Nakatani,Y., Dent,S.Y.R. and Almouzni,G. (2009) The HP1alpha-CAF1-SetDB1-containing complex provides H3K9me1 for Suv39-mediated K9me3 in pericentric heterochromatin. *EMBO Rep.*, **10**, 769–775.

146. Al-Sady,B., Madhani,H.D. and Narlikar,G.J. (2013) Division of labor between the chromodomains of HP1 and Suv39 methylase enables coordination of heterochromatin spread. *Mol. Cell*, **51**, 80–91.

147. Mishima,Y., Jayasinghe,C.D., Lu,K., Otani,J., Shirakawa,M., Kawakami,T., Kimura,H., Hojo,H., Carlton,P., Tajima,S. *et al.* (2015) Nucleosome compaction facilitates HP1γ binding to methylated H3K9. *Nucleic Acids Res.*, **43**, 10200–10212.

148. Maison,C., Bailly,D., Quivy,J.-P. and Almouzni,G. (2016) The methyltransferase Suv39h1 links the SUMO pathway to HP1α marking at pericentric heterochromatin. *Nat. Commun.*, **7**, 12224.

149. Cardinale,S., Bergmann,J.H., Kelly,D., Nakano,M., Valdivia,M.M., Kimura,H., Masumoto,H., Larionov,V. and Earnshaw,W.C. (2009) Hierarchical inactivation of a synthetic human kinetochore by a chromatin modifier. *Mol. Biol. Cell*, **20**, 4194–4204.

150. Ohzeki,J., Shono,N., Otake,K., Martins,N.M.C., Kugou,K., Kimura,H., Nagase,T., Larionov,V., Earnshaw,W.C. and Masumoto,H. (2016) KAT7/HBO1/MYST2 regulates CENP-A chromatin assembly by antagonizing Suv39h1-Mediated centromere inactivation. *Dev. Cell*, **37**, 413–427.

151. Huang,C., Cheng,J., Bawa-Khalfe,T., Yao,X., Chin,Y.E. and Yeh,E.T.H. (2016) SUMOylated ORC2 recruits a histone demethylase to regulate centromeric histone modification and genomic stability. *Cell Rep.*, **15**, 147–157.

152. Black,J.C., Manning,A.L., Van Rechem,C., Kim,J., Ladd,B., Cho,J., Pineda,C.M., Murphy,N., Daniels,D.L., Montagna,C. *et al.* (2013) KDM4A lysine demethylase induces site-specific copy gain and rereplication of regions amplified in tumors. *Cell*, **154**, 541–555.

153. Chen,X., Skutt-Kakaria,K., Davison,J., Ou,Y.-L., Choi,E., Malik,P., Loeb,K., Wood,B., Georges,G., Torok-Storb,B. *et al.* (2012) G9a/GLP-dependent histone H3K9me2 patterning during human hematopoietic stem cell lineage commitment. *Genes Dev.*, **26**, 2499–2511.

154. Shinkai,Y. and Tachibana,M. (2011) H3K9 methyltransferase G9a and the related molecule GLP. *Genes Dev.*, **25**, 781–788.

155. Seum,C., Bontron,S., Reo,E., Delattre,M. and Spierer,P. (2007) Drosophila G9a is a nonessential gene. *Genetics*, **177**, 1955–1957.

156. Kramer,J.M., Kochinke,K., Oortveld,M.A.W., Marks,H., Kramer,D., de Jong,E.K., Asztalos,Z., Westwood,J.T., Stunnenberg,H.G., Sokolowski,M.B. *et al.* (2011) Epigenetic regulation of learning and memory by Drosophila EHMT/G9a. *PLoS Biol.*, **9**, e1000569.

157. Stabell,M., Eskeland,R., Bjørkmo,M., Larsson,J., Aalen,R.B., Imhof,A. and Lambertsson,A. (2006) The Drosophila G9a gene encodes a multi-catalytic histone methyltransferase required for normal development. *Nucleic Acids Res.*, **34**, 4609–4621.

158. Jih,G., Iglesias,N., Currie,M.A., Bhanu,N.V., Paulo,J.A., Gygi,S.P., Garcia,B.A. and Moazed,D. (2017) Unique roles for histone H3K9me states in RNAi and heritable silencing of transcription. *Nature*, **547**, 463–467.

159. Zee,B.M., Levin,R.S., Xu,B., LeRoy,G., Wingreen,N.S. and Garcia,B.A. (2010) In vivo residue-specific histone methylation dynamics. *J. Biol. Chem.*, **285**, 3341–3350.

160. Margueron,R. and Reinberg,D. (2011) The Polycomb complex PRC2 and its mark in life. *Nature*, **469**, 343–349.

161. Jung,H.R., Pasini,D., Helin,K. and Jensen,O.N. (2010) Quantitative mass spectrometry of histones H3.2 and H3.3 in Suz12-deficient mouse embryonic stem cells reveals distinct, dynamic post-translational modifications at Lys-27 and Lys-36. *Mol. Cell. Proteomics*, **9**, 838–850.

162. Ferrari,K.J., Scelfo,A., Jammula,S., Cuomo,A., Barozzi,I., Stützer,A., Fischle,W., Bonaldi,T. and Pasini,D. (2014) Polycomb-dependent H3K27me1 and H3K27me2 regulate active transcription and enhancer fidelity. *Mol. Cell*, **53**, 49–62.

163. Juan,A.H., Wang,S., Ko,K.D., Zare,H., Tsai,P.-F., Feng,X., Vivanco,K.O., Ascoli,A.M., Gutierrez-Cruz,G., Krebs,J. *et al.* (2016) Roles of H3K27me2 and H3K27me3 examined during fate specification of embryonic stem cells. *Cell Rep.*, **17**, 1369–1382.

164. Streubel,G., Watson,A., Jammula,S.G., Scelfo,A., Fitzpatrick,D.J., Oliviero,G., McCole,R., Conway,E., Glancy,E., Negri,G.L. *et al.* (2018) The H3K36me2 Methyltransferase Nsd1 Demarcates PRC2-Mediated H3K27me2 and H3K27me3 Domains in Embryonic Stem Cells. *Mol. Cell*, **70**, 371–379.

165. Tachibana,M., Sugimoto,K., Fukushima,T. and Shinkai,Y. (2001) Set domain-containing protein, G9a, is a novel lysine-preferring mammalian histone methyltransferase with hyperactivity and specific selectivity to lysines 9 and 27 of histone H3. *J. Biol. Chem.*, **276**, 25309–25317.

166. Ikegami,K., Iwatani,M., Suzuki,M., Tachibana,M., Shinkai,Y., Tanaka,S., Greally,J.M., Yagi,S., Hattori,N. and Shiota,K. (2007) Genome-wide and locus-specific DNA hypomethylation in G9a deficient mouse embryonic stem cells. *Genes Cells*, **12**, 1–11.

167. Wu,H., Chen,X., Xiong,J., Li,Y., Li,H., Ding,X., Liu,S., Chen,S., Gao,S. and Zhu,B. (2011) Histone methyltransferase G9a contributes to H3K27 methylation in vivo. *Cell Res.*, **21**, 365–367.

168. Guo,A.-S., Huang,Y.-Q., Ma,X.-D. and Lin,R.-S. (2016) Mechanism of G9a inhibitor BIX-01294 acting on U251 glioma cells. *Mol. Med. Rep.*, **14**, 4613–4621.

169. Giunta,S. and Funabiki,H. (2017) Integrity of the human centromere DNA repeats is protected by CENP-A, CENP-C, and CENP-T. *Proc. Natl. Acad. Sci. U.S.A.*, **114**, 1928–1933.

170. Zeitlin,S.G., Baker,N.M., Chapados,B.R., Soutoglou,E., Wang,J.Y.J., Berns,M.W. and Cleveland,D.W. (2009) Double-strand DNA breaks recruit the centromeric histone CENP-A. *Proc. Natl. Acad. Sci. U.S.A.*, **106**, 15762–15767.

171. Aleksandrov,R., Dotchev,A., Poser,I., Krastev,D., Georgiev,G., Panova,G., Babukov,Y., Danovski,G., Dyankova,T., Hubatsch,L. *et al.* (2018) Protein dynamics in complex DNA lesions. *Mol. Cell*, **69**, 1046–1061.

172. Helfricht,A., Wiegant,W.W., Thijssen,P.E., Vertegaal,A.C., Luijsterburg,M.S. and van Attikum,H. (2013) Remodeling and spacing factor 1 (RSF1) deposits centromere proteins at DNA double-strand breaks to promote non-homologous end-joining. *Cell Cycle*, **12**, 3070–3082.

173. Tomonaga,T., Matsushita,K., Yamaguchi,S., Oohashi,T., Shimada,H., Ochiai,T., Yoda,K. and Nomura,F. (2003) Overexpression and mistargeting of centromere protein-A in human primary colorectal cancer. *Cancer Res.*, **63**, 3511–3516.

174. Au,W.-C., Crisp,M.J., DeLuca,S.Z., Rando,O.J. and Basrai,M.A. (2008) Altered dosage and mislocalization of histone H3 and Cse4p lead to chromosome loss in Saccharomyces cerevisiae. *Genetics*, **179**, 263–275.

175. Gascoigne,K.E., Takeuchi,K., Suzuki,A., Hori,T., Fukagawa,T. and Cheeseman,I.M. (2011) Induced ectopic kinetochore assembly bypasses the requirement for CENP-A nucleosomes. *Cell*, **145**, 410–422.

176. Drané,P., Ouararhni,K., Depaux,A., Shuaib,M. and Hamiche,A. (2010) The death-associated protein DAXX is a novel histone chaperone involved in the replication-independent deposition of H3.3. *Genes Dev.*, **24**, 1253–1265.

177. Daniel Ricketts,M., Frederick,B., Hoff,H., Tang,Y., Schultz,D.C., Singh Rai,T., Grazia Vizioli,M., Adams,P.D. and Marmorstein,R. (2015) Ubinuclein-1 confers histone H3.3-specific-binding by the HIRA histone chaperone complex. *Nat. Commun.*, **6**, 7711.

178. Elsässer,S.J., Huang,H., Lewis,P.W., Chin,J.W., Allis,C.D. and Patel,D.J. (2012) DAXX envelops a histone H3.3–H4 dimer for H3.3-specific recognition. *Nature*, **491**, 560–565.

179. Lewis,P.W., Elsaesser,S.J., Noh,K.-M., Stadler,S.C. and Allis,C.D. (2010) Daxx is an H3.3-specific histone chaperone and cooperates with ATRX in replication-independent chromatin assembly at telomeres. *Proc. Natl. Acad. Sci. U.S.A.*, **107**, 14075–14080.

180. Rapkin,L.M., Ahmed,K., Dulev,S., Li,R., Kimura,H., Ishov,A.M. and Bazett-Jones,D.P. (2015) The histone chaperone DAXX maintains the structural organization of heterochromatin domains. *Epigenet. Chromatin*, **8**, 44.

181. Filipescu,D., Naughtin,M., Podsypanina,K., Lejour,V., Wilson,L., Gurard-Levin,Z.A., Orsi,G.A., Simeonova,I., Toufektchan,E., Attardi,L.D. *et al.* (2017) Essential role for centromeric factors following p53 loss and oncogenic transformation. *Genes Dev.*, **31**, 463–480.

182. Castillo,A.G., Pidoux,A.L., Catania,S., Durand-Dubief,M., Choi,E.S., Hamilton,G., Ekwall,K. and Allshire,R.C. (2013) Telomeric repeats facilitate CENP-A(Cnp1) incorporation via telomere binding proteins. *PLoS One*, **8**, e69673.

183. Goldberg,A.D., Banaszynski,L.A., Noh,K.-M., Lewis,P.W., Elsaesser,S.J., Stadler,S., Dewell,S., Law,M., Guo,X., Li,X. *et al.* (2010) Distinct factors control histone variant H3.3 localization at specific genomic regions. *Cell*, **140**, 678–691.

184. Michod,D., Bartesaghi,S., Khelifi,A., Bellodi,C., Berliocchi,L., Nicotera,P. and Salomoni,P. (2012) Calcium-dependent dephosphorylation of the histone chaperone DAXX regulates H3.3 loading and transcription upon neuronal activation. *Neuron*, **74**, 122–135.

185. Voon,H.P.J., Hughes,J.R., Rode,C., De La Rosa-Velázquez,I.A., Jenuwein,T., Feil,R., Higgs,D.R. and Gibbons,R.J. (2015) ATRX plays a key role in maintaining silencing at interstitial heterochromatic loci and imprinted genes. *Cell Rep.*, **11**, 405–418.

186. Udugama,M., Chang,F.T.M., Chan,F.L., Tang,M.C., Pickett,H.A., McGhie,J.D.R., Mayne,L., Collas,P., Mann,J.R. and Wong,L.H. (2015) Histone variant H3.3 provides the heterochromatic H3 lysine 9 tri-methylation mark at telomeres. *Nucleic Acids Res.*, **43**, 10227–10237.

187. Chow,C.-M., Georgiou,A., Szutorisz,H., Maia e Silva,A., Pombo,A., Barahona,I., Dargelos,E., Canzonetta,C. and Dillon,N. (2005) Variant histone H3.3 marks promoters of transcriptionally active genes during mammalian cell division. *EMBO Rep.*, **6**, 354–360.

188. Jin,C., Zang,C., Wei,G., Cui,K., Peng,W., Zhao,K. and Felsenfeld,G. (2009) H3.3/H2A.Z double variant-containing nucleosomes mark 'nucleosome-free regions' of active promoters and other regulatory regions. *Nat. Genet.*, **41**, 941–945.

189. Chen,P., Zhao,J., Wang,Y., Wang,M., Long,H., Liang,D., Huang,L., Wen,Z., Li,W., Li,X. *et al.* (2013) H3.3 actively marks enhancers and primes gene transcription via opening higher-ordered chromatin. *Genes Dev.*, **27**, 2109–2124.

190. Tomonaga,T., Matsushita,K., Ishibashi,M., Nezu,M., Shimada,H., Ochiai,T., Yoda,K. and Nomura,F. (2005) Centromere protein H is up-regulated in primary human colorectal cancer and its overexpression induces aneuploidy. *Cancer Res.*, **65**, 4683–4689.

191. Biermann,K., Heukamp,L.C., Steger,K., Zhou,H., Franke,F.E., Guetgemann,I., Sonnack,V., Brehm,R., Berg,J., Bastian,P.J. *et al.* (2007) Gene expression profiling identifies new biological markers of neoplastic germ cells. *Anticancer Res.*, **27**, 3091–3100.

192. Amato,A., Schillaci,T., Lentini,L. and Di Leonardo,A. (2009) CENPA overexpression promotes genome instability in pRb-depleted human cells. *Mol. Cancer*, **8**, 119.

193. Wu,Q., Qian,Y.-M., Zhao,X.-L., Wang,S.-M., Feng,X.-J., Chen,X.-F. and Zhang,S.-H. (2012) Expression and prognostic significance of centromere protein A in human lung adenocarcinoma. *Lung Cancer*, **77**, 407–414.

194. Vardabasso,C., Hasson,D., Ratnakumar,K., Chung,C.-Y., Duarte,L.F. and Bernstein,E. (2014) Histone variants: emerging players in cancer biology. *Cell. Mol. Life Sci.*, **71**, 379–404.

195. Thompson,S.L., Bakhoum,S.F. and Compton,D.A. (2010) Mechanisms of chromosomal instability. *Curr. Biol.*, **20**, R285–95.

196. Bastians,H. (2015) Causes of Chromosomal Instability. In: *Recent Results in Cancer Research*. Vol. **200**, pp. 95–113.

197. Barra,V. and Fachinetti,D. (2018) The dark side of centromeres: types, causes and consequences of structural abnormalities implicating centromeric DNA. *Nat. Commun.*, **9**, 4340.

198. Waghray,A., Schober,M., Feroze,F., Yao,F., Virgin,J. and Chen,Y.Q. (2001) Identification of differentially expressed genes by serial analysis of gene expression in human prostate cancer. *Cancer Res.*, **61**, 4283–4286.

199. Kwan,P.S., Lau,C.C., Chiu,Y.T., Man,C., Liu,J., Tang,K.D., Wong,Y.C. and Ling,M.-T. (2013) Daxx regulates mitotic progression and prostate cancer predisposition. *Carcinogenesis*, **34**, 750–759.

200. Pan,W.-W., Zhou,J.-J., Liu,X.-M., Xu,Y., Guo,L.-J., Yu,C., Shi,Q.-H. and Fan,H.-Y. (2013) Death domain-associated protein DAXX promotes ovarian cancer development and chemoresistance. *J. Biol. Chem.*, **288**, 13620–13630.

201. Tsourlakis,M.C., Schoop,M., Plass,C., Huland,H., Graefen,M., Steuber,T., Schlomm,T., Simon,R., Sauter,G., Sirma,H. *et al.* (2013) Overexpression of the chromatin remodeler death-domain-associated protein in prostate cancer is an independent predictor of early prostate-specific antigen recurrence. *Hum. Pathol.*, **44**, 1789–1796.

202. Puto,L.A., Brognard,J. and Hunter,T. (2015) Transcriptional repressor DAXX promotes prostate cancer tumorigenicity via suppression of autophagy. *J. Biol. Chem.*, **290**, 15406–15420.

203. Chen,L.-Y. and Chen,J.D. (2003) Daxx silencing sensitizes cells to multiple apoptotic pathways. *Mol. Cell. Biol.*, **23**, 7108–7121.

204. Bush,K.M., Yuen,B.T.K., Barrilleaux,B.L., Riggs,J.W., O'Geen,H., Cotterman,R.F. and Knoepfler,P.S. (2013) Endogenous mammalian histone H3.3 exhibits chromatin-related functions during development. *Epigenet. Chromatin*, **6**, 7.

205. Jang,C.-W., Shibata,Y., Starmer,J., Yee,D. and Magnuson,T. (2015) Histone H3.3 maintains genome integrity during mammalian development. *Genes Dev.*, **29**, 1377–1392.

206. Jiao,Y., Shi,C., Edil,B.H., de Wilde,R.F., Klimstra,D.S., Maitra,A., Schulick,R.D., Tang,L.H., Wolfgang,C.L., Choti,M.A. *et al.* (2011) DAXX/ATRX, MEN1, and mTOR pathway genes are frequently altered in pancreatic neuroendocrine tumors. *Science*, **331**, 1199–1203.

207. Wu,G., Broniscer,A., McEachron,T.A., Lu,C., Paugh,B.S., Becksfort,J., Qu,C., Ding,L., Huether,R., Parker,M. *et al.* (2012) Somatic histone H3 alterations in pediatric diffuse intrinsic pontine gliomas and non-brainstem glioblastomas. *Nat. Genet.*, **44**, 251–253.

208. Schwartzentruber,J., Korshunov,A., Liu,X.-Y., Jones,D.T.W., Pfaff,E., Jacob,K., Sturm,D., Fontebasso,A.M., Quang,D.-A.K., Tönjes,M. *et al.* (2012) Driver mutations in histone H3.3 and

chromatin remodelling genes in paediatric glioblastoma. *Nature*, **482**, 226–231.

209. Weinberg,D.N., Allis,C.D. and Lu,C. (2017) Oncogenic mechanisms of histone H3 mutations. *Cold Spring Harb. Perspect. Med.*, **7**, a026443.

210. Yuen,B.T.K. and Knoepfler,P.S. (2013) Histone H3.3 Mutations: A Variant Path to Cancer. *Cancer Cell*, **24**, 567–574.

211. Voon,H.P.J., Udugama,M., Lin,W., Hii,L., Law,R.H.P., Steer,D.L., Das,P.P., Mann,J.R. and Wong,L.H. (2018) Inhibition of a K9/K36 demethylase by an H3.3 point mutation found in paediatric glioblastoma. *Nat. Commun.*, **9**, 3142.

212. Sturm,D., Witt,H., Hovestadt,V., Khuong-Quang,D.-A., Jones,D.T.W., Konermann,C., Pfaff,E., Tönjes,M., Sill,M., Bender,S. *et al.* (2012) Hotspot mutations in H3F3A and IDH1 define distinct epigenetic and biological subgroups of glioblastoma. *Cancer Cell*, **22**, 425–437.

213. Lovejoy,C.A., Li,W., Reisenweber,S., Thongthip,S., Bruno,J., de Lange,T., De,S., Petrini,J.H.J., Sung,P.A., Jasin,M. *et al.* (2012) Loss of ATRX, genome instability, and an altered DNA damage response are hallmarks of the alternative lengthening of telomeres pathway. *PLoS Genet.*, **8**, e1002772.

214. Heaphy,C.M., de Wilde,R.F., Jiao,Y., Klein,A.P., Edil,B.H., Shi,C., Bettegowda,C., Rodriguez,F.J., Eberhart,C.G., Hebbar,S. *et al.* (2011) Altered telomeres in tumors with ATRX and DAXX mutations. *Science*, **333**, 425.

215. Sakellariou,D., Chiourea,M., Raftopoulou,C. and Gagos,S. (2013) Alternative lengthening of telomeres: recurrent cytogenetic aberrations and chromosome stability under extreme telomere dysfunction. *Neoplasia*, **15**, 1301–1313.