Temporal control of epigenetic centromere specification

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Abstract

All living organisms require accurate mechanisms to fatefully inherit their genetic material during cell division. The centromere is a unique locus on each chromosome that supports a multiprotein structure called the kinetochore. During mitosis, the kinetochore is responsible for connecting chromosomes to spindle microtubules, allowing faithful segregation of the duplicated genome. In most organisms, centromere position and function is not defined by the local DNA sequence context but rather by an epigenetic chromatin-based mechanism. Centromere protein A (CENP-A) is central to this process, as chromatin assembled from this histone H3 variant is essential for assembly of the centromere complex as well as for its epigenetic maintenance. As a major determinant of centromere function, CENP-A assembly requires tight control, both in its specificity for the centromere and in timing of assembly. In the last few years there have been several new insights in the molecular mechanism that allow this process to occur. We will review these here and discuss the general implications for the way we understand the mechanism of cell division.

Introduction

Cell division requires the coordinate action of mechanisms that duplicate the full complement of the genome and subsequently partition these copies among newly formed daughter cells. Key to the latter process of chromosome segregation is a chromosomal region called the centromere. This region, initially defined cytologically as the primary constriction on metazoan metaphase chromosomes, is the site of assembly of a large complex of proteins that function in tethering sister chromatids together as well as in generating attachments to spindle microtubules in a bipolar fashion (Figure

1). Microtubule binding is mediated by the kinetochore, a complex of proteins that is assembled onto the centromere complex during mitosis.

Centromeres are propagated largely independent of cis-acting DNA sequences indicating they are maintained through an epigenetic mechanism. We will discuss recent evidence implicating the unique chromatin structure that is generated by the histone H3 variant CENP-A (centromere protein A) in epigenetic maintenance of centromeres. In addition, we will discuss work in both fly and human cells that has shed light on the temporal dynamics of CENP-A chromatin. Determining how centromeric chromatin turns over across the cell division cycle is crucial to understand how CENP-A, as a key component of the epigenetic mechanism at centromeres, is itself inherited and duplicated. We will present recent findings and outline differences and commonalities among different organisms. Finally, we will discuss the consequences of the unique timing of centromeric chromatin assembly for maintenance of centromere function and architecture.

The role of DNA sequence in centromere specification.

Contrary to what is observed in *S. cerevisiae*, where a specific 125 bp sequence is sufficient for centromere formation, in most eukaryotes the centromere typically forms on a subset of long arrays of repetitive DNA. In humans, such DNA regions are comprised of repetitive arrays of 171 bp sequences called α-satellites (or alphoid DNA), that can span several megabases (Schueler and Sullivan, 2006). One of the first centromeric proteins described, Centromere Protein B (CENP-B) was found to bind specifically to those repeats suggesting that this DNA sequence is important for centromere function (Masumoto et al., 1989). While maintenance of pre-existing centromeres appears largely independent of the underlying DNA sequence (discussed

below), a critical role for centromeric DNA was uncovered in attempts to generate centromeres *de novo*, both in fission yeast and human cells (Takahashi et al., 1992; Harrington et al., 1997). In human cells, it was further shown that both alphoid DNA and the presence of CENP-B binding sites are important for *de novo* centromere formation (Ohzeki et al., 2002). However, apart from the mammalian CENP-B box, identifying what sequence features of centromeric DNA are important for centromere formation has been challenging, principally because the highly repetitive tandem sequence repeats present in metazoan centromeres are not conserved among species or, in the case of e.g. human centromeres, even among different chromosomes of an individual organism (Willard, 1991). This suggests that the role of DNA sequences at the centromere is to provide a structural context rather than a specific sequence motif.

The Epigenetic inheritance of centromeres

Despite its requirement for centromere establishment, there is little support for a prominent role of centromeric DNA in the maintenance of existing centromeres. Evidence against such role came initially from the characterization of stably transmitted dicentric chromosomes in humans and flies. Two active centromeres on a single chromosome may be deleterious to the cell, leading to chromosome breakage and loss. However, dicentric chromosomes can be inherited by inactivating one of the centromeres, without changing its DNA sequence (Earnshaw and Migeon, 1985), in a process that is still not fully understood. This indicates that canonical centromere sequences are not sufficient for centromere function. In addition to insufficiency, multiple lines of evidence have demonstrated that centromeric DNA is also not necessary for the maintenance of centromeres. While CENP-B binding sites are critical for *de novo* formation of centromeres, the CENP-B protein is non-essential in mice,

indicating that as long as preassembled centromeres are inherited, CENP-B binding to centromeres is not required (Hudson et al., 1998; Kapoor et al., 1998; Perez-Castro et al., 1998).

The most direct evidence demonstrating centromere maintenance in the absence of specific DNA sequence elements *in cis* was provided by the discovery of human neocentromeres which are formed in genomic regions lacking any characteristic centromeric DNA sequences (Voullaire et al., 1993). At such neocentromeres, all known essential centromere and kinetochore proteins (except CENP-B) are associated with non-repetitive, non-alphoid DNA. Since its discovery almost twenty years ago, around one hundred unique neocentromeres have been described (Marshall et al., 2008). While neocentromeres often occur on the same chromosome arm (e.g. the q-arms of chromosome 3, 13 and 15), fine-mapping of centromere proteins at 3 neocentromeres on 13q32 revealed that formation of centromeric chromatin occurred on different DNA sequences (Alonso et al., 2003). The absence of true "hotspots" for binding of the centromeric histone, CENP-A, highlights the independence of centromere formation from the DNA sequence. Neocentromeres are stably transmitted during mitosis and in some cases are even germ-line transmitted (Tyler-Smith et al., 1999). These observations led to the proposal that centromere function and propagation is not dependent on any specific DNA sequence, but instead is mediated through an epigenetic mechanism (Vafa and Sullivan, 1997; Warburton et al., 1997).

Neocentromere formation is not limited to *Homo sapiens*. They have been described in a wide range of organisms. In *Candida albicans*, following the replacement of the centromere of chromosome 5 with a reporter gene, cells were able to maintain this chromosome by forming neocentromeres either distally, at loci around 200 to 450 kb

away from where the reporter is located, or immediately adjacent to the insertion (Ketel et al., 2009). Similarly, in *S. pombe*, in which cen I (centromere from chromosome I) is deleted, neocentromeres have also been identified (Ishii et al., 2008). ChIP-chip analysis of centromere proteins Mis6 and CENP-A showed that neocentromeres were frequently formed either proximal to the left or the right telomere close to, but not in contact with, subtelomeric heterochromatin (Ishii et al., 2008). Interestingly, in *Drosophila*, centromere formation can be driven by overexpression of CENP-A. These ectopic centromeres have the propensity to form adjacent to heterochromatin domains, suggesting a role for heterochromatin in neocentromere formation (Olszak et al., 2011). However, such a role does not appear to be universally required for centromere formation as, at least a subset, of human neocentromeres appear to lack appreciable heterochromatin (Alonso et al., 2010).

CENP-A: A key epigenetic centromere mark

The epigenetic mode of centromere transmission finds its basis in a unique local chromatin structure. Early experiments in fission yeast addressing the behavior of gene insertions in the centromere in the presence of Trichostatin A (TSA) - an inhibitor of histone deacetylases – showed that upon TSA addition and consequent high levels of histone acetylation, centromere silencing was alleviated and centromere function was impaired. This chromatin state at the centromere was found to be inherited throughout cell divisions, even in the absence of TSA, suggesting that the assembly of functional centromeres is at least partially imprinted in local chromatin (Ekwall et al., 1997).

One of the fundamental elements that differentiates the centromere from the rest of the chromosome is the presence of CENP-A, a histone H3 variant, that replaces the canonical histone H3.1 in nucleosomes at active centromeres (Palmer et al., 1991).

CENP-A is a well conserved protein that has been identified in a broad range of eukaryotes, including *S. cerevisiae* (Cse4), *S. pombe* (Cnp1), *C. elegans* (HCP-3), *D. melanogaster* (CID), *A. Thaliana* (CenH3), *X. laevis*, *G. gallus* and *H. Sapiens* (CENP-A). CENP-A is critical for the formation of the centromere/kinetochore complex (Foltz et al., 2006) and depletion of CENP-A, in all organisms tested so far, leads to loss of many centromere/kinetochore proteins and failure in chromosome segregation (Howman et al., 2000; Régnier et al., 2005; Liu et al., 2006).

The conserved role of this histone variant and its constitutive localization at all active centromeres throughout the cell cycle make CENP-A a strong candidate for a major function in centromere specification and propagation (Allshire and Karpen, 2008; Silva and Jansen, 2009). Indeed, recent studies provide direct evidence that CENP-A acts as a seed that is both necessary and sufficient for the formation of a fully functional centromere (Barnhart et al., 2011; Mendiburo et al., 2011). In *Drosophila* S2 cells, tethering CENP-A^{CID}-LacI fusion protein to stably integrated LacO arrays leads to the formation of functional ectopic centromeres. Furthermore ectopic CENP-A^{CID} was capable of assembling a functional kinetochore and direct normal CENP-A^{CID} (lacking the LacI-anchor) binding to the ectopic site. A similar approach was used in human U2OS cells to tether HJURP (Holliday Junction Recognizing Protein), a CENP-A specific chaperone necessary for CENP-A assembly (Dunleavy et al., 2009; Foltz et al., 2009). In this study, LacO targeted HJURP resulted in the stable recruitment of CENP-A to the array, functional kinetochore formation and stable kinetochore-microtubule attachments (Barnhart et al., 2011). In these tethering experiments, the presence of two centromeres (the endogenous and the ectopic one) led to mitotic failure, preventing the analysis of the long-term fate of the ectopic centromere. To address this, Mendiburo *et*

al, employed a plasmid-based artificial chromosome containing LacO arrays to assess the heritability of ectopic centromeres across cell divisions in *Drosophila* S2 cells (Mendiburo et al., 2011). Normally, these plasmids undergo DNA replication but cannot segregate accurately, and are therefore lost within several cell cycles. Similar to the chromosomal LacO insertions described above, tethering CENP-A^{CID}-LacI fusion protein to the LacO arrays on those plasmids led to the recruitment of centromeric proteins and to microtubule binding. Critically, these ectopic centromeres promote stable transmission of the plasmids for multiple cell divisions, even in the absence of the fusion protein that initiated centromere formation. Together, these results provide direct evidence that CENP-A is sufficient to direct centromere formation and can initiate a self-perpetuating epigenetic feedback mechanism that controls centromere identity and stable inheritance (Figure 2). In agreement with these findings, CENP-A nucleosomes are remarkably stable and turn over only upon replication by redistribution to the two sister chromatids (Régnier et al., 2005; Jansen et al., 2007; Hemmerich et al., 2008). Such stability provides further evidence for a key role for CENP-A in the maintenance of centromere identity.

With CENP-A taking center stage in the propagation of the centromere through an epigenetic mechanism, what then, is the role of alphoid DNA in centromere formation and maintenance? While no clear role has been ascribed, it is interesting to note that inactive human alphoid arrays at chromosomally integrated sites show a variegating low level of CENP-A (Nakano et al., 2003) suggesting that alphoid DNA may be a preferred substrate for CENP-A containing chromatin. This suggests that, while DNA sequence or a particular sequence context is dispensable for maintenance of CENP-A *per se*, alphoid DNA may facilitate centromere maintenance at multigenerational timescales.

CENP-A assembly and cell cycle control

Current evidence is consistent with a role of CENP-A in epigenetically specifying centromere identity through a chromatin-based positive feedback mechanism. This suggests that tight coordination must exist between the duplication of centromeric DNA, replication of centromeric chromatin and cell division. Interestingly, contrary to canonical histones, in human cells, CENP-A loading does not occur during S-phase (Shelby et al., 2000; Jansen et al., 2007). Direct evidence for the timing of assembly of nascent CENP-A came from SNAP-based fluorescent pulse labeling experiments that allowed visualization of the newly synthesized pool of CENP-A. Despite constitutive CENP-A expression under these conditions, assembly into centromeric chromatin occurs only after exit form mitosis, in early G1 phase. In agreement, measurements of steady state CENP-A across the cell cycle and photo-bleaching experiments assessing CENP-A exchange rates also established that the protein is a stable centromeric component that loads upon mitotic exit (Schuh et al., 2007; Hemmerich et al., 2008). Several factors that control the temporal loading of CENP-A have been identified to date. One of them, Mis18, was first identified in fission yeast and was shown to be required for CENP-A localization (Hayashi et al., 2004). In humans, a complex of proteins that includes the Mis18 homologues Mis18α and Mis18β, as well as Mis18BP1^{HsKNL2}, has been identified that is essential for CENP-A loading at the centromere (Fujita et al., 2007). Moreover, all three proteins show a similar temporal localization pattern: targeting to the centromere just prior to CENP-A assembly during anaphase and leaving around two to three hours later, in mid G1(Fujita et al., 2007; Maddox et al., 2007; Silva and Jansen, 2009). Despite their critical role in CENP-A assembly, none of these proteins appear to interact directly with CENP-A (Hayashi et al., 2004; Fujita et al., 2007; Lagana

et al., 2010), suggesting an indirect role for this complex. The fact that the Mis18 complex arrives prior to CENP-A assembly led to the proposal that it works as a licensing factor for CENP-A assembly, perhaps by altering its surrounding chromatin environment. Changing global acetylation levels by treatment of cells with TSA, a histone deacetylase inhibitor was reported to suppress a Mis18 defect, suggesting that the Mis18 complex changes local acetylation status. In support of this idea, recent work has shown that CENP-A assembly and functional centromere formation on alphoid DNA that also bears Tet operator sequences can be stimulated by local TetR-mediated tethering of Mis18 or by tethering of histone acetyl transferases (Ohzeki et al., 2012). One possible model is that the Mis18 complex includes a, yet to be defined, histone acetyl transferase that modifies local chromatin in a way that renders it permissive for CENP-A assembly.

Another crucial factor for CENP-A assembly is its chaperone, Scm3 in fission yeast and its homologue HJURP in human cells (Dunleavy et al., 2009; Foltz et al., 2009; Pidoux et al., 2009; Sanchez-Pulido et al., 2009; Williams et al., 2009). HJURP co-localizes with CENP-A at centromeres during late telophase/early G1, and has the capacity to promote CENP-A chromatin assembly *in vitro* (Barnhart et al., 2011). Recent work has shown that the Mis18 complex is required for HJURP recruitment to the centromere, and that artificial tethering of HJURP to a chromosomal site is able to promote CENP-A loading in the absence of Mis18 (Barnhart et al., 2011). This suggests that Mis18 functions to allow HJURP access to the centromere. However, the molecular details of such a role are yet to be defined. Contrary to the interaction reported between fission yeast Scm3 and Mis18 (Pidoux et al., 2009; Williams et al., 2009), in human cells a direct interaction between HJURP and Mis18 complex has not been described. Interestingly, the RbAp46/48

proteins have been found to interact with both the Mis18 complex and HJURP (Dunleavy et al., 2009; Lagana et al., 2010) providing a possible link between these two key CENP-A assembly factors.

More recently, other players that participate in CENP-A assembly have been identified: Rsf-1, a member of the ATP-dependent chromatin remodeling and spacing factor (RSF) complex, and MgcRacGAP (Perpelescu et al., 2009; Lagana et al., 2010). Like the Mis18 complex and HJURP these novel players localize at the centromere in a cell cycle dependent manner but do so later, during mid and late G1 phase respectively. This suggests they are involved in a different step of the CENP-A assembly process that appears drawn out over the length of G1 phase.

Cell cycle control of CENP-A assembly

While many of the components of the CENP-A assembly machinery have been identified, how they recognize centromeres specifically and how they are controlled in the cell cycle is unclear. Recently, we and others have made advances on this latter point. Timing of assembly of new CENP-A coincides with the conclusion of mitosis, but the mitotic signal that initiates centromere propagation remained unidentified. Several models have been proposed implicating mitotic events in CENP-A assembly that include microtubule mediated forces, kinetochore formation, nuclear architecture or destruction of an inhibitor of CENP-A assembly (Mellone and Allshire, 2003; Carroll and Straight, 2006; Jansen et al., 2007; Schuh et al., 2007; Allshire and Karpen, 2008).

SNAP-based pulse-chase labeling allows for the specific visualization of newly synthesized CENP-A, which has been employed to demonstrate G1 phase-restricted timing of CENP-A assembly (Jansen et al., 2007). Using this methodology, we have

recently dissected the mechanism of cell cycle control of CENP-A assembly and shown that timing of CENP-A loading at centromeres is controlled by cyclin-dependent kinases (Cdks) (Silva et al., 2012). Cdks are serine/threonine protein kinases that, through phosphorylation of a large number of downstream targets, are responsible for driving key events of the cell cycle. Cdk function depends on their association with specific cyclins that confer both activity and substrate specificity (Pagliuca et al., 2011). While Cdk levels remain relatively constant, cyclins oscillate during the cell cycle, driven by waves of cyclin gene expression and targeted protein destruction. Interphase cyclins are ubiquitinated by the Skp1/Cul1/F-box protein (SCF) complex, while mitotic cyclins are targeted for degradation by the anaphase-promoting complex/cyclosome (APC/C) during mitotic exit. This combination of Cdk phosphorylation and cyclin degradation allows for a proper sequence of cell cycle events in a unidirectional manner (Pines and Hagan, 2011).

Constitutive expression of CENP-A throughout the cell cycle does not override G1 phase restricted assembly indicating that assembly is actively prevented in S, G2 and mitotic phases of the cell cycle (Jansen et al., 2007). During these stages, the cell cycle is controlled primarily by Cdk2/cyclin A and Cdk1/cyclin B complexes. A key observation from our work has been that inhibition of Cdk activity using pan-Cdk inhibitors such as Roscovitine are sufficient to drive stable assembly of CENP-A prior to mitosis in human cells (Silva et al., 2012). These results rule out a specific involvement of mitotic events in signaling CENP-A assembly, except for the down regulation of Cdk activity during mitotic exit. Importantly, this process is independent of APC/C mediated proteolysis and protein synthesis indicating that turning on CENP-A assembly does not require synthesis or destruction of a specific CENP-A assembly activator or inhibitor,

respectively. Furthermore, chicken DT40 cells carrying defined mutations in Cdk1 and Cdk2 lose cell cycle control of CENP-A assembly, leading to CENP-A loading throughout the cell cycle. Not only do these experiments corroborate the initial results using broad Cdk inhibitors, they also demonstrate that Cdk1 and Cdk2 are sufficient to maintain CENP-A assembly at bay.

CENP-A assembly factors such as HJURP or members of the Mis18 complex are localized to the centromere in a cell cycle dependent fashion (Fujita et al., 2007; Maddox et al., 2007; Dunleavy et al., 2009; Foltz et al., 2009). Like CENP-A assembly, the timing of localization of these assembly factors can be manipulated by controlling Cdk activity. Most known CENP-A assembly factors are phosphorylated *in vivo* (Olsen et al., 2006; Dephoure et al., 2008; Wang et al., 2008; Mayya et al., 2009). Strikingly, mutants in Mis18BP1^{HsKNL2} that prevent its phosphorylation are targeted to centromere prematurely, prior to mitosis. This observation, along with the role of Cdk1 and Cdk2 in maintaining cell cycle control has led to a model in which temporal regulation of CENP-A assembly is achieved by Cdk dependent phosphorylation of CENP-A assembly factor(s), preventing their localization to the centromere, consequently impeding CENP-A assembly outside G1 (Figure 3). While Mis18BP1^{HsKNL2} has emerged as a likely target of control, additional players are possibly involved as Mis18BP1^{HsKNL2} presence at G2 phase centromeres is not sufficient to promote CENP-A assembly (Silva et al., 2012).

Conservation and differences of temporal control among eukaryotes.

Loading of CENP-A homologues across different species occurs in a cell cycle regulated fashion. In *S. pombe*, using a temperature sensitive allele of a fluorescently-tagged version of CENP-A^{Cnp1}, it was shown that CENP-A^{Cnp1} re-incorporation following a shift to the permissive temperature occurs during two discrete cell cycle phases: early S and,

to a lesser extent, G2 phase (Takayama et al., 2008). While interpretation of experiments based on such ts-alleles is difficult, they suggest that CENP-A is assembled primarily upon mitotic exit (that in fission yeast is immediately followed by S phase), consistent with observations in human cells. Interestingly, Scm3, a key HJURP-like factor in fission yeast, is phosphorylated at a putative Cdk consensus site (Pidoux et al., 2009). It is possible that such phosphorylation event takes place during mitosis, coinciding with loss of Scm3 from the centromere. Like fission yeast, *Drosophila melanogaster* syncytial embryos also miss a G1 phase (Lee and Orr-Weaver, 2003), and CENP-A^{CID} assembly occurs in a tight window during anaphase. As is the case in human cells, CENP-A^{CID} assembly is strictly dependent on exit from mitosis (Schuh et al., 2007). Interestingly, and in apparent contrast to the studies described above, in *Drosophila* S2 and Kc167 cells, assembly of CENP-A^{CID} occurs in metaphase (Mellone et al., 2011). Cdk1 activity peaks in metaphase indicating that possibly, in this case, the role of Cdks in blocking CENP-A^{CID} assembly is not conserved. Another possibility is that the cyclin/Cdk complexes involved in cell cycle restricting CENP-A^{CID} assembly are different in these cells. Cyclin A is degraded in early mitosis (den Elzen and Pines, 2001; Geley et al., 2001; Huang and Raff, 2002) while Cdk1 activity in prometaphase and metaphase is maintained by cyclin B. Possibly, CENP-A^{CID} assembly in *Drosophila* is maintained in an inhibitory state exclusively by cyclin A driven Cdk activities during S and G2 phases, initiating the onset of CENP-A^{CID} assembly in prometaphase. Consistently, Cal1, a CENP-A^{CID} assembly factor in flies, targets to centromeres at this stage in mitosis. Furthermore, the Mellone et al study demonstrated that CENP-A^{CID} assembly can be prevented by expression of non-degradable cyclin A. Although the cell cycle regulation of CENP-A^{CID} appears to be different between *Drosophila* cell lines and syncytial embryos, control may nevertheless be limited to Cdk-cyclin A in both systems. However,

cyclin A degradation may be differentially regulated in these two cell types. In agreement with this, in *Drosophila* syncytial embryos, cyclin B is only partially degraded upon mitotic exit and is not essential for mitosis (Knoblich and Lehner, 1993; Raff et al., 2002). Cyclin B is therefore an unlikely candidate to play a critical role in controlling the timing of centromere propagation. Instead, mitotic exit may be regulated primarily by loss Cdk-cyclin A activity. Cyclin A is essential for mitosis and its levels are maintained until anaphase during the initial mitotic divisions of the D. melanogaster syncytial embryo (Lehner and O'Farrell, 1989) potentially preventing CENP-A^{CID} assembly until mitotic exit in this system. While this awaits a definitive test, these observations appear consistent with a differential role of cyclin A and B in maintaining cell cycle control of CENP-A assembly in *Drosophila*.

Cell cycle control of the CCAN

A number of proteins, commonly referred to as the constitutive centromere-associated network (CCAN) associate with CENP-A chromatin throughout the cell cycle and are considered core components maintaining centromere structure (Foltz et al., 2006; Okada et al., 2006; Cheeseman and Desai, 2008). While dependent on CENP-A chromatin for their recruitment to centromere (Liu et al., 2006), they in turn can influence assembly of nascent CENP-A. Examples of such components are CENP-H, CENP-I and CENP-K, three proteins that are present as a complex and are required to recruit newly synthesized CENP-A to centromeres (Okada et al., 2006), possibly by recruitment of the FACT1 and CHD1 chromatin modulators, that are in turn necessary for CENP-A to be properly assembled. CENP-N and CENP-C, also CCAN components, have been found to directly bind to CENP-A nucleosomes *in vitro* and play a role in the recruitment of nascent CENP-A to centromeres *in vivo*, placing them within the positive

epigenetic feedback loop responsible for centromere propagation (Carroll et al., 2009, 2010). While to date no other component of the CCAN has displayed the G1 phase restricted assembly pattern as observed for CENP-A, the assembly of several CCAN components is under cell cycle control. CENP-I has been shown to be stable throughout the cell cycle, except during S phase where a proportion of centromeric CENP-I is turned over, possibly reflecting deposition of nascent CENP-I onto newly replicated centromeres (Hemmerich et al., 2008). In contrast, CENP-N molecules undergo rapid exchange during G1 phase of the cell cycle, at the time of CENP-A assembly, becoming stably associated to centromeres only after mid S phase (Hellwig et al., 2011), when centromeric DNA is replicated. CENP-C, another CENP-A interacting factor (Carroll et al., 2010) is also stably bound to the centromere during mitosis and S phase (Hemmerich et al., 2008). Recently, four CCAN proteins, CENP-T, CENP-W, CENP-S and CENP-X were found to form a complex, functionally and structurally resembling a histone heterotetramer that interacts with and partially wraps DNA *in vitro* (Nishino et al., 2012). When tethered on a naïve chromatin locus, CENP-T along with CENP-C are sufficient to drive kinetochore formation (Gascoigne et al., 2011), indicating that the CENP-T-W-S-X complex, together with CENP-C, form the essential kinetochore forming platform. Analysis of dynamics of the CENP-T/W sub complex reveals a dynamics opposite to that of e.g. CENP-N, with rapid exchange in late S-phase and G2 while remaining immobile in G1 phase (Prendergast et al., 2011).

In summary, although the functional role of these divergent dynamic states across the cell cycle remains unclear, what emerges from these studies is that while the CCAN is constitutive at steady state, different components or sub-complexes display different local dynamics. These differences can be orders of magnitude apart, from immobile

proteins that last days to hours (CENP-A, -I) to highly dynamic proteins such as CENP-N during G1 phase or CENP-T and -X during G2 phase that exchange on a second to minute timescale.

Importance of the cell cycle control of CENP-A assembly

Despite the fact that CENP-A assembly is tightly cell cycle regulated, the reasons for such control are still unclear. One possible explanation is that this mechanism allows for a temporal separation of centromeric chromatin assembly from general chromatin assembly. It has been shown that when CENP-A expression is limited to S-phase, it is uniformly distributed throughout chromatin (Shelby et al., 1997), indicating that CENP-A can be assembled by the general chromatin assembly machinery. Limiting CENP-A assembly to outside S-phase can therefore help restrict it to the centromere. In fission yeast, CENP-A assembly occurs in early S phase. However, CENP-A gene synthesis precedes bulk H3 synthesis and centromeres are early replicating, potentially facilitating centromere specific deposition of CENP-A even in a system where assembly is overlapping with S phase (Castillo et al., 2007).

G1 phase assembly and subsequent centromeric DNA replication in S phase creates a condition where during mitosis, the centromeres carry only half of the maximum CENP-A complement. It has been proposed (Jansen et al., 2007) and recently shown (Dunleavy et al., 2011) that histone H3 is assembled among CENP-A containing nucleosomes during S phase. It is possible that a mixture of CENP-A/H3 chromatin is important for kinetochore formation and chromosome segregation. The recent finding that CCAN components such as the CENP-T-W-S-X complex can make chromatin contacts independent of CENP-A, either directly to DNA (Gascoigne et al., 2011), or in the context of H3 chromatin (Hori et al., 2008) fuels this idea. However, whether such H3 chromatin

is functionally relevant remains to be tested. In this respect it is important to note that pre-S phase assembly of CENP-A is not universal. As in a subset of fission yeast cells, measurement of CENP-A^{CENH3} levels in plant centromeres indicates a G2 phase mode of assembly, inconsistent with the idea of a delicate S phase generated CENP-A/H3 balance. Nevertheless, some form of cell cycle coupling of CENP-A assembly appears to be universal, indicating the necessity for maintaining a balance between cell division and epigenetic centromere propagation. In this respect it is noteworthy that temporal control of CENP-A assembly in vertebrates by the Cdk cycle constitutes an on/off mechanisms in which the turning "on" in early G1 and "off" in late G1 may help to restrict CENP-A assembly to a narrow cell cycle window and in turn maintain centromere size.

Outlook

The recent flurry in discoveries that provide a more complete understanding on how centromeres work and how they are regulated during the cell cycle have raised many novel questions and left others unanswered. How is centromeric chromatin marked for CENP-A assembly by Mis18 complex members and how can CENP-A, once assembled survive through chromatin disruption during DNA replication? With respect to cell cycle control questions include: What is the functional role of cell cycle regulation of CCAN assembly and what is the significance of G1 phase restricted assembly of CENP-A? What are the consequences of CENP-A targeting to the centromere outside G1 phase? Moreover, despite the prominent role of Cdk activity in timing CENP-A assembly, the molecular mechanism requires further dissection. Mis18BP1^{HsKNL2} appears to be a part of this mechanism of temporal control (Silva et al., 2012) but how its activity is

regulated and to which other CENP-A assembly factors this control extends are questions of great interest in future efforts.

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Abbreviations

CENP – centromere protein

Cdk – cyclin dependent kinase

TSA - Trichostatin A

HJURP - Holliday Junction Recognizing Protein

CCAN - constitutive centromere-associated network

Rsf – remodeling and spacing factor

MGCRacGAP - Male germ cell Rac GTPase-activating protein

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Figures

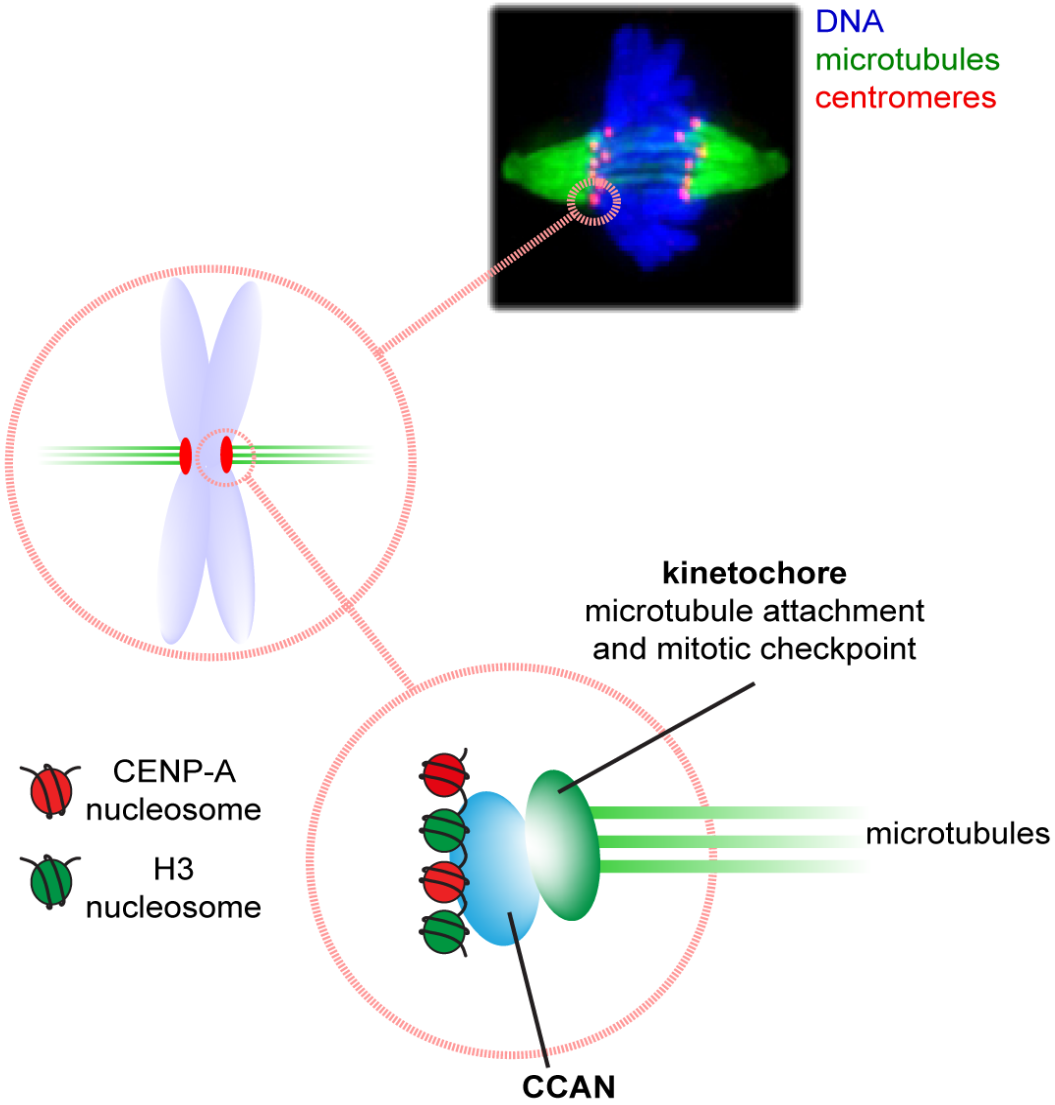

Figure 1. The centromere, a chromatin locus essential for chromosome segregation. Centromeres are nucleoprotein complexes that form on a single locus on each chromosome and form the attachment site for spindle microtubules. Centromeric chromatin is distinguished from general chromatin by the presence of CENP-A containing nucleosomes, which are interspersed with histone H3 nucleosomes. CENP-A chromatin is the site of assembly of the constitutive centromere-associated network (CCAN), which in turn nucleates the kinetochore, a group of proteins with microtubule binding activity and proteins required for mitotic checkpoint signaling. This complex organization allows for proper chromosome segregation by ensuring bipolar attachment of the two sister chromatids during metaphase and drives separation of sisters during anaphase.

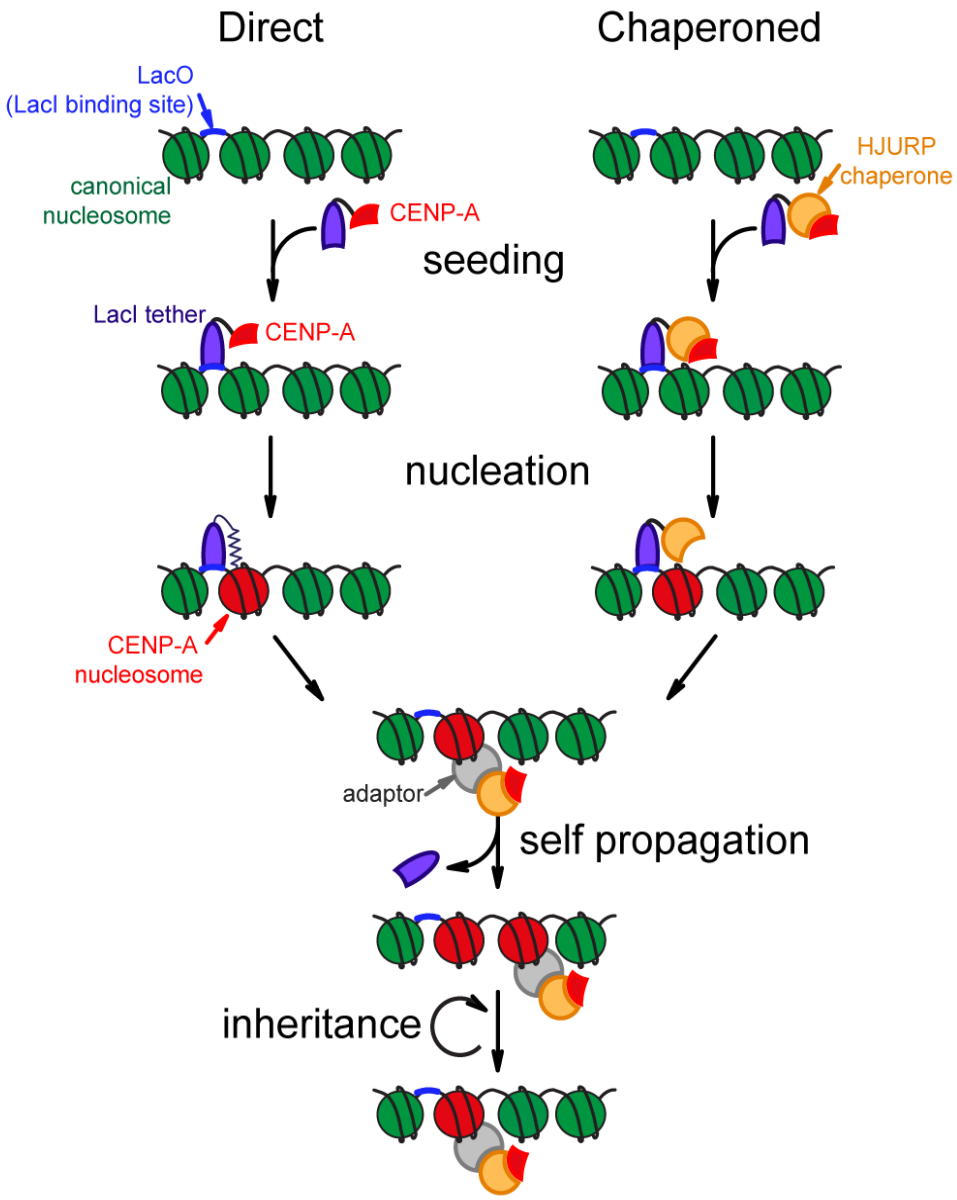

Figure 2. Seeding and epigenetic propagation of centromeres. CENP-A is targeted to a naive LacO marked chromatin locus either by direct fusion (CENP-A^{CID} in Drosophila S2 cells (Mendiburo et al., 2011) to LacI or through recruitment by the LacI-tethered chaperone HJURP (human CENP-A in U2OS cells (Barnhart et al., 2011). Tethering of CENP-A results in the nucleation of CENP-A chromatin. This in turn leads to functional centromere and kinetochore formation and triggers the propagation of CENP-A chromatin in a self-templating manner (likely through an adaptor intermediate) without the need for the initial LacI-seed. Once formed, CENP-A chromatin propagation and turnover through cell divisions reach an equilibrium resulting in stable inheritance of this epigenetic centromere mark.

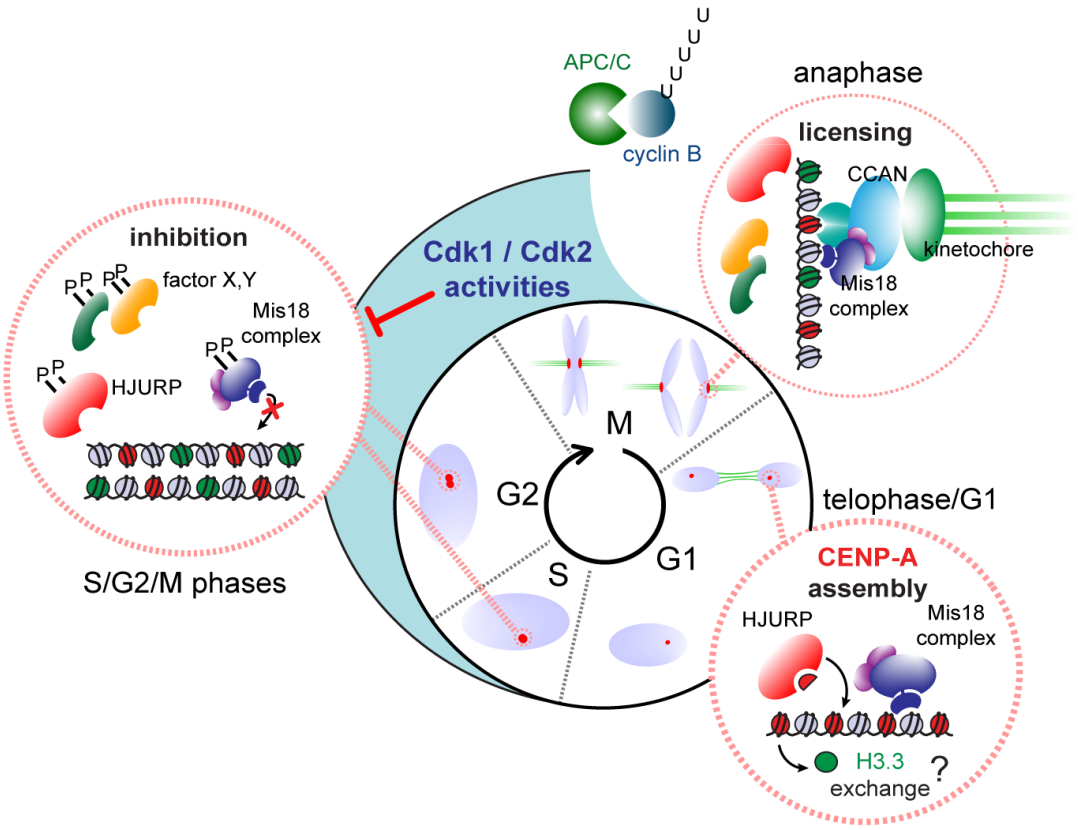

Figure 3. Model for cell cycle control of CENP-A assembly. Cdk1 and Cdk2 activities maintain the CENP-A assembly machinery in an inactive non-centromeric state through phosphorylation of Mis18BP1HsKNL2 and possibly other components. Cartoon illustrates Cdk1/Cdk2-mediated inhibition of CENP-A assembly, accomplished in part through phosphorylation (P) of Mis18BP1HsKNL2 (member of the Mis18 complex) during S, G2 and M phases. Phosphorylation of HJURP and or other unknown factors (symbolized with X,Y) may also be required for this inhibition. Inhibition is alleviated trough APC/C mediated destruction of cyclin B and consequent loss of Cdk1 activity in anaphase. This allows targeting of the Mis18 complex to the centromere in anaphase (licensing) followed by HJURP targeting and CENP-A assembly in G1 phase. CENP-A assembly in G1 phase possibly involves an exchange with H3.3 (Dunleavy et al., 2011). Canonical (H3.1 containing) nucleosomes are shown in light blue, H3.3 nucleosomes in green and CENP-A nucleosomes in red.