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Epigenetics Regulate Centromere Formation and Kinetochore Function

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Abstract The eukaryote centromere was initially defined cytologically as the primary constriction on vertebrate chromosomes and functionally as a chromosomal feature with a relatively low recombination frequency. Structurally, the centromere is the foundation for sister chromatid cohesion and kinetochore formation. Together these provide the basis for interaction between chromosomes and the mitotic spindle, allowing the efficient segregation of sister chromatids during cell division. Although centromeric (CEN) DNA is highly variable between species, in all cases the functional centromere forms in a chromatin domain defined by the substitution of histone H3 with the centromere specific H3 variant centromere protein A (CENP-A), also known as CENH3. Kinetochore formation and function are dependent on a variety of regional epigenetic modifications that appear to result in a loop chromatin conformation providing exterior CENH3 domains for kinetochore construction, and interior heterochromatin domains essential for sister chromatid cohesion. In addition pericentric heterochromatin provides a structural element required for spindle assembly checkpoint function. Advances in our understanding of CENH3 biology have resulted in a model where kinetochore location is specified by the epigenetic mark left after dilution of CENH3 to daughter DNA strands during S phase. This results in a self-renewing and self-reinforcing epigenetic state favorable to reliably mark centromere location, as well as to provide the optimal chromatin configuration for kinetochore formation and function. J. Cell. Biochem. 104: 2027–2039, 2008.

Key words: centromere; kinetochore; epigenetics; histone modification; CENH3

Reliable partitioning of the genome during cell division is the basis for all higher unicellular and multi-cellular life. This function depends on the centromere, a region of unique chromatin composition found at the primary constriction of eukaryote chromosomes that is essential for the segregation of sister chromatids during cell division. Centromeric (CEN) chromatin forms a base upon which the kinetochore is assembled during entry into mitosis. The kinetochore is a massive multi-protein assembly that mediates microtubule attachment between the centromere and mitotic spindle. A functional kinetochore is essential for accurate chromosome segregation and thus genetic stability. Kinetochores sense errors in chromosome attachment to the mitotic spindle, activating the spindle assembly checkpoint (SAC) to delay metaphase-to-anaphase transition until all chromosomes achieve bipolar spindle attachment. Tension applied to sister kinetochores by the correct attachment of microtubules (MTs) is sensed mechanically and inactivates the checkpoint. These functions become unstable during oncogenesis resulting in segregation defects, aneuploidy, and oncogenic progression.

In all species studied the centromere is defined by a domain of unique chromatin containing the centromere specific, H3 histone variant, centromere protein A (CENP-A), now referred to as CENH3. In higher animals this domain is buried in a large pericentric heterochromatin domain deficient in CENH3. In contrast the DNA upon which the centromere forms is highly variable between species, ranging from the simple ~125-bp centromere of *Saccharomyces cerevisiae* to the highly repetitive α satellite sequences of primates. Although these sequences influence the positioning of the

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centromere, the ability in humans of rare functional CENH3 containing neocentromeres to form outside of these α-satellite DNA sequences provides strong indirect evidence that epigenetic mechanisms dictate centromere formation.

FEATURES OF THE CENTROMERE AND COMPARISON BETWEEN SPECIES

CENH3, the key non-DNA component of centromeric chromatin, is conserved across vast species while the size and sequence of underlying centromeric DNA is extremely variable and rapidly evolving. The simple point centromere found in fission yeast *S. cerevisiae* forms on a small ~125 bp stretch of centromeric DNA and functions as a base for a small kinetochore that binds a single microtubule. In organisms with a diffuse centromere, such as the nematode *Caenorhabditis elegans*, kinetochores form along the entire chromosome. In comparison most eukaryotes have more complex holocentric centromeres consisting of long stretches of repetitive DNA. The best studied is that of *Schizosaccharomyces pombe* where the sequence of CEN DNA from all three chromosomes is known [Wood et al., 2002]. The centromeres of this species has a non-repetitive 4–7 kb long central sequence (cnt) surrounded by centromere-specific innermost repeats (imr), upon which the kinetochore is assembled. In the flanking pericentric region are long tandem arrays of outer repeats (otr) common between the three chromosomes [Blackwell et al., 2004]. In primates, all centromeres examined contain α satellite DNA elements. This is a satellite DNA family based on divergent 170-bp monomers arranged in a tandem, head-to-tail fashion resulting in an overall directionality. In Human chromosomes, centromeric DNA contains megabase long arrays of tandemly repeated 171-bp α-satellite DNA [Schueler et al., 2001]. Within the surrounding pericentric heterochromatin region, similar non-repeating satellite monomers are found. DNA repeats comprise the primary sequence of all complex centromeres in both plants and animals.

In humans, many α-satellite repeats contain a conserved 17 bp binding site for the sequence-specific DNA binding protein centromere protein B (CENP-B). Since mutation of the CENP-B binding site reduced the efficiency of mammalian artificial chromosome formation on synthetic alphoid arrays [Ohzeki et al., 2002], it was originally suggested that recruiting of sequence-specific DNA-binding proteins to α-satellite DNA defined centromere location. However CENP-B is not required for the formation of a functional centromere and under normal conditions, alphoid DNA is not sufficient for the recruitment of many essential kinetochore proteins, such as CENH3, CENP-C, and CENP-E [Sullivan and Schwartz, 1995; Warburton, 2001; Nakano et al., 2003]. The de novo formation of centromeres was recently shown to require CENP-B, which also functions to establish heterochromatin domains on repeats of CENP-B binding sites in the presence of a functional centromere elsewhere on the chromosome [Okada et al., 2007]. However, because mice lacking functional CENP-B are normal exhibiting unaltered centromere and kinetochore structure and function [Kapoor et al., 1998], the result implies that epigenetic inheritance, rather than a sequence-directed mechanism, is sufficient for centromere formation and function during normal development.

In humans neocentromeres can form outside of regions containing α-satellite DNA and CENP-B boxes, and these misplaced centromeres generally assemble fully functional kinetochores that are stably inherited through many cell divisions [Slater et al., 1993]. Finally, when constructing human artificial chromosomes with α-satellite and non α-satellite DNA, centromeres were formed that were able to spread over non α-satellite DNA [Lam et al., 2006]. The migration of centromeres and the existence of neocentromeres outside of α-satellite DNA in humans provide convincing evidence that centromere specification is determined by an epigenetic rather than a sequence-specific mechanism [Warburton, 2001]. Although repetitive DNA sequences are not essential for centromere formation they provide an environment favorable for self-assembly of a heritable, stable centromere as shown by the ability of α-satellite containing human artificial chromosomes to incorporate CENH3 and form de novo functional kinetochores when introduced into human cell lines [Masumoto et al., 2004]. This is likely due to the ability of these sequences to package into condensed heterochromatin domains and not due to actual DNA base pair sequence. It is the composition of the chromatin and its conformation, and not the underlying DNA sequence,

which is important for specifying a functional centromere.

EPIGENETIC MODIFICATIONS AND THEIR FUNCTIONS

Epigenetics is the study of heritable changes in gene function that occur without a change in the DNA sequence. Epigenetic mechanisms are mediated through chromatin structure. The basic unit of chromosome structure, the nucleosome, partitions the DNA into units of approximately 200 bp in length. At the molecular level, each chromosome is a repeat of nucleosomes and shorter segments of DNA that link the individual nucleosomes. The nucleosome core particle comprises 147 bp of DNA that make 1.75 turns around the outer surface of a protein octamer assembled from a tetramer of histones H3 and H4 and 2 dimers of histones H2A and H2B. The linking DNA is associated with a fifth histone, histone H1, which binds DNA as it enters and exits the nucleosome stabilizing 2 complete turns of the DNA around the histone octamer.

Histone proteins are the substrate for post-translational modifications which form the basis for many epigenetic effects. Collectively, the core histones are the targets of several different types of post-translational modifications. These include ADP-ribosylation, ubiquitination, phosphorylation, acetylation, and methylation and involve more than 40 different amino acid residues. The majority of these modifications occur within the N-terminal tails of the core histones. It is these post-translational modifications, which encode most of the epigenetic information specifying chromatin structure and function [Kouzarides, 2007]. Most modifications function in the regulation of gene expression by modulating chromatin conformation and access of the DNA sequence to regulatory proteins [Sims et al., 2003] and through specific interactions with chromatin binding proteins. In the nucleus, chromatin is found in a loose, transcription permissive, euchromatin form and in a condensed heterochromatin form generally found to repress transcription. Heterochromatin formation and maintenance is correlated with a number of epigenetic modifications including reduced acetylation of the N-terminal lysines of histones H3 and H4, trimethylation of lysine 20 on histone H4, trimethylation of lysine 9 on histone H3, and trimethylation of lysine 27 on histone H3. In comparison euchromatin is associated with histone lysine acetylation, methylation of histone H3 on lysine 4 and methylation of H3 on lysine 36 [Cheung and Lau, 2005]. The function of some post-translational histone modifications, particularly methylations, is thought to be conferred through protein domains found in many key regulatory factors. The best characterized are domains that specifically recognize and bind acetylated (bromodomain) [Zeng and Zhou, 2002] or methylated (chromodomain) histones [Cavalli and Paro, 1998]. For example the epigenetic mark trimethylation of lysine 9 on histone H3 is found in regions of heterochromatin were it provides a binding site for the chromodomain of heterochromatic protein 1 (HP1). Cross-linking of heterochromatin through the dimerization of HP1 is thought to stabilize and promote a compact structure. HP1 is enriched at the centromeres of nearly all eukaryotic chromosomes and has a role in the recruitment of cohesin complexes to the centromere which are required to bind sister chromatids prior to segregation [Bernard et al., 2001].

The incorporation of histone variants into chromatin and their subsequent post-translational modification represents an additional epigenetic mechanism [Cheung and Lau, 2005]. For example CENH3 is a histone H3 variant whose incorporation marks centromere position, but it can also be phosphorylated by Aurora A or Aurora B kinase, a modification that is needed for enrichment of Aurora B at the centromere and for proper kinetochore function [Kunitoku et al., 2003].

Lastly, epigenetic effects can be mediated by direct methylation of DNA at cytosine-phosphate-guanine sites (CpG) by DNA methyltransferases [Lettini et al., 2007]. These epigenetic marks are found to be highly heritable and their critical function in the cell is demonstrated by the lethality of DNA methyltransferase knockout in mice. A role for this type of epigenetic modification in centromere and kinetochore function is illustrated in the rare genetic disease IMMUNODEFICIENCY–CENTROMERIC instability–facial dysmorphism (ICG) syndrome ([Jiang et al., 2005]. Centromeric instability is the most typical feature of the disease. All patients show abnormal hypomethylation of the classical DNA satellites 2 and 3 and of α-satellite DNA, each of which are major components of constitutive

heterochromatin found in the centromeric and pericentric region respectively. The resulting defect in heterochromatin formation and chromosome segregation most grossly affects chromosomes 1, 9, and 16 where astonishing anomalies exist including whole-arm deletions, multibranched chromosomes, translocations, and isochromosomes. A subgroup of these patients is shown to have mutations to DNMT3B, a gene encoding a de novo DNA methyltransferase. Although the role of DNA methylation on centromere location and kinetochore function has not been investigated experimentally, ICG syndrome underscores the importance of this epigenetic mark, especially on proper heterochromatin formation, and confirms the critical role heterochromatin has in proper centromere location, kinetochore formation and chromosome segregation.

CENH3 AND OTHER EPIGENETIC MODIFICATIONS ASSOCIATED WITH THE CENTROMERE

Centromeric chromatin is defined by the unique incorporation of the histone H3 variant CENH3. The importance of CENH3 to centromere and kinetochore function is shown by numerous studies in budding yeast, *C. elegans*, fission yeast, mouse, *Drosophila*, and human cells were mutation of CENH3 results in a failure of chromosomes to properly segregate. CENH3 mutants generally display disrupted centromeric chromatin, a disrupted kinetochore, severely defective chromosome segregation and fail to recruit many kinetochore components to the centromere in interphase and mitosis. These results are corroborated by the discovery that CENH3 substitutes for H3 in active CEN and neocentromeric nucleosomes, but is not present at the inactive centromere found in pseudo-dicentric chromosomes [Warburton, 2001]. CENH3 chromatin directly recruits a six-component CENP-A nucleosome- associated complex (CENP-ANAC) which regulates the assembly of other downstream centromere components and the kinetochore during mitosis [Foltz et al., 2006]. Together this evidence shows that CENH3 provides an epigenetic mark that specifies centromere position, and that its incorporation forms a substrate for kinetochore construction.

When the distribution of CENH3 and H3 were analyzed on linearized chromatin fibers from HeLA and Drosophilia cells, CENH3 was found incorporated as 10–40 kb long nucleosome clusters interspersed with H3 containing nucleosomes. Analysis of the posttranslational modification status of H3 showed that it was hypoacetylated, which is a marker associated with heterochromatin but also enriched in dimethylated lysine 9 (diMe-K4 H3), a marker usually associated with euchromatin [Sullivan and Karpen, 2004].

The centromeric chromatin region containing CENH3 is flanked by stretches of pericentric heterochromatin which also has a role in proper kinetochore formation and accurate segregation of chromosomes during mitosis. A number of factors affect the assembly and inheritance of pericentric heterochromatin including the RNA interference (RNAi) machinery, methylation of lysine 9 on histone H3(Me-K9 H3) and its interaction with the chromodomain of HP1 [Grewal and Moazed, 2003]. The role of HP1 is evident in *S. pombe* were cells that lack Swi6 (the HP1 homologue) are deficient in pericentric heterochromatin, are unable to recruit the cohesin subunit Rad21 to centromeres and fail to maintain centromere cohesion [Nonaka et al., 2002]. In mouse cell lines lacking Su(var)3-9 and thus the Me-K9 H3 epigenetic marker, HP1 no longer associates with pericentric heterochromatin and cohesion is lost between pericentric regions of sister chromatids [Guenatri et al., 2004]. Deletion of the Su (var)3-9 methyltransferase in flies and mammals, or the deletion of the Dicer ribonuclease in mammals, disrupts pericentric heterochromatin and causes defects in chromosome cohesion [Blower and Karpen, 2001; Fukagawa et al., 2004; Guenatri et al., 2004]. Using engineered minichromosomes, it was recently reported that intact heterochromatin and RNAi interference mechanisms are also essential to initially establish CENH3 at the centromere of fission yeast [Folco et al., 2008]. A summary of the epigenetic modifications associated with the centromere and their probable function is found in Table I.

THE CENH3-CONTAINING NUCLEOSOME

Reconstitution experiments were used to demonstrate that CENH3 forms a rigid interface with histone H4. This rigid characteristic and the ability to target to the centromere was dependent on loop1 and the α2 helix of CENH3,

TABLE I. Epigenetic Modifications Associated With the Centromere and Their Functions

Modification	Domain	Function
CENH3 incorporation	Centromeric DNA	Defines heritable centromere location Substrate for kinetochore construction Phasing with H3 important in loop conformation
Methylation of lysine 9 on histone H3 (MeK9 H3)	Pericentric heterochromatin	Provides HP1 binding site Chromatin compaction Cohesin accumulation Provides structural integrity
Methylation of lysine 20 on histone H4 (MeK20 H4)	Pericentric heterochromatin	Chromatin compaction Provides structural integrity
Methylation of lysine 4 on histone H3 (diMeK4 H3)	Centromeric chromatin	Euchromatin marker found in CEN chromatin Involved in loop conformation Marker for replacement by CENH3 in G1?
Hypoacetylation	Centromeric and Pericentric heterochromatin	Dense chromatin structure Heterochromatin integrity
Phosphorylation	CENH3	Required for Aurora B accumulation at the centromere
Methylation of DNA	Centromeric and Pericentric satellite DNA	Heterochromatin condensation Heterochromatin integrity Provides structural integrity?

termed the CENP-A centromere targeting domain, or CATD. Transplantation of this structure into canonical histone H3 conferred the rigid characteristic and allowed the hybrid histone to replace CENH3 in centromere localization and functional kinetochore formation. These studies concluded that the CENH3 nucleosome is a more compact version of the regular octomeric nucleosome with CENH3 in place of H3 [Schueler and Sullivan, 2006; Black et al., 2007]. This finding has recently been questioned by studies in Drosophilia where isolation and characterization of CENH3 containing nucleosomes at interphase found that the functional CENH3 nucleosome occurs as a tetramer of one molecule each of CenH3, H4, H2A, and H2B, referred to as a hemisome [Dalal et al., 2007]. The authors suggest that this hemisome structure may be universal for CenH3 nucleosomes. If confirmed, this finding will have radical implications on our understanding of CENH3 function, nucleosome structure, and higher-order chromatin formation. Their model suggests that the asymmetric nature of the hemisome interferes with the liquid crystal like array of packaged nucleosomes during chromosome compaction forcing the unpackaged CENH3 hemisome to the outside portion of the centromere domain. This mechanism is suggested to provide a CENH3 nucleosome base plate for kinetochore formation and a dense inner domain specialized for chromatid cohesion.

A number of groups have previously proposed similar "loop" mechanisms for centromere formation [Schueler and Sullivan, 2006; Black et al., 2007]. In the model proposed by Schueler and Sullivan (Fig. 1a) subdomains containing CENH3 nucleosomes are interspersed with H3 dimethylated at lysine 4 (H3K4me2) containing nucleosomes across a percentage of the megabase α-satellite repeats with the remaining areas containing unmodified H3 containing nucleosomes. The remainder of the α-satellite DNA is assembled into pericentric heterochromatin (purple) that flanks the CEN chromatin domain. During chromosome condensation at metaphase (Fig. 1b), the interspersed domains are thought to promote coiling of the DNA so that stacks of CENH3 nucleosomes form a base plate on the exterior of the chromosome where they can interact with kinetochore initiation factors while the H3 containing nucleosomes associate between the sister kinetochores. In this model pericentric heterochromatin containing the H3-K9 methylation marker is assembled into a distinct domain which may force the CENH3 base plate to the outside of the structure, as well as providing a site for cohesin accumulation and sister chromatid binding [Schueler and Sullivan, 2006]. Each of these loop models suggest that, once in place, the epigenetic markers associated with the centromere function in the assembly of the mature kinetochore tri-laminar plate.

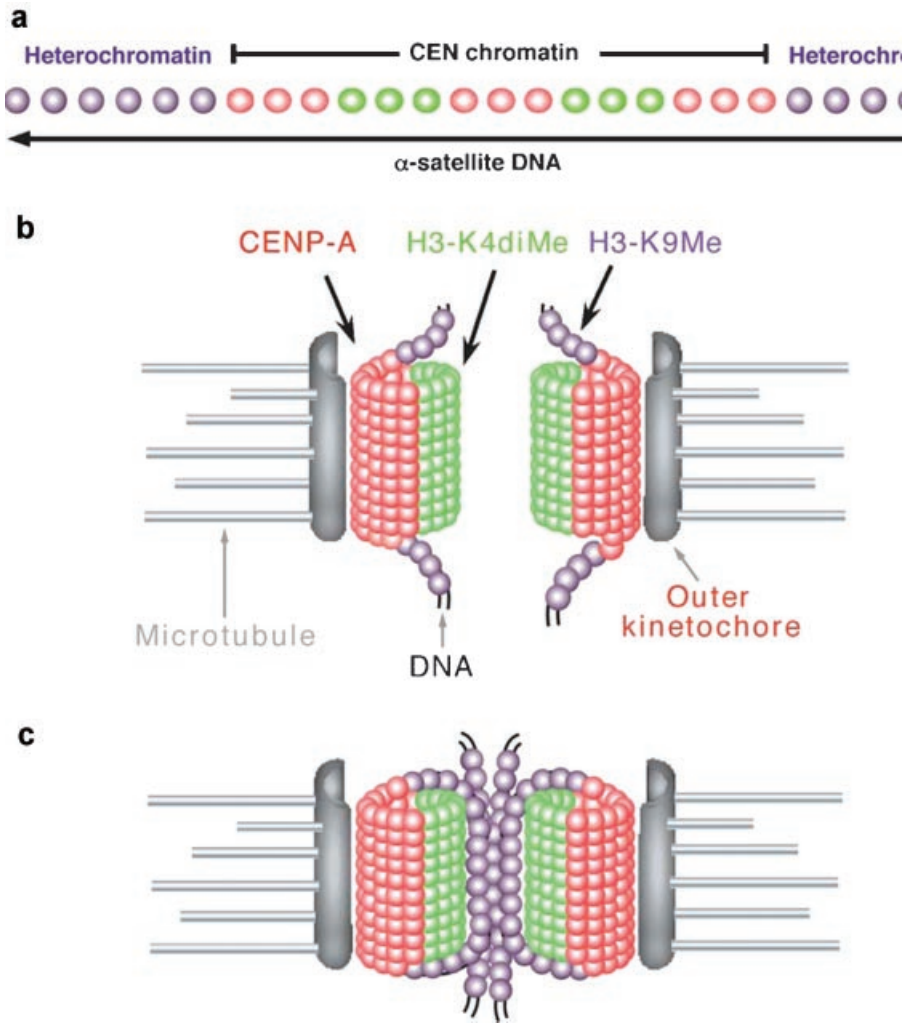

Fig. 1. Unique organization of centromere regions in humans. **a**: On linear, two-dimensional chromatin fibers, subdomains of nucleosomes containing centromeric (CEN) histone CENP-A (red) are interspersed with H3 dimethylated at lysine 4 (H3K4me2) (green) to form a domain of CEN chromatin on a fraction of the megabase regions of human α-satellite DNA. The remainder of the α-satellite DNA is assembled into heterochromatin (purple) that flanks one or both sides of CEN chromatin domain. **b**: At metaphase, when mitotic chromosomes condense, the interspersed domains promote coiling of the DNA so that stacks of CENP-A nucleosomes are presented to the poleward face of the chromosome where they can interact with other kinetochore proteins. H3-containing nucleosomes are oriented between sister kinetochores. **c**: Heterochromatin defined by nucleosomes containing H3-K9 methylation (purple) is assembled into a domain that is distinct from CEN chromatin. Higher-order packaging of heterochromatin between sister kinetochores may promote orientation of CENP-A, pushing it toward the outside of the chromosome. Heterochromatin in this region is also important for recruiting cohesion proteins that are sustained at the centromere until chromatid separation at anaphase. Reproduced from Schueler and Sullivan (2006).

LOADING OF CENH3 INTO THE CENTROMERE

Fujita et al. have identified hMis18alpha, hMis18beta, and M18BP1 as factors that prime the human centromere for CENH3 recruitment. These three proteins accumulate at the telophase-G1 centromere and RNAi knockdown of any of the three abolishes recruitment of newly synthesized CENH3 and results in defective chromosome segregation [Fujita et al., 2007]. Analyses of the determinants required for the loading of CENH3 into centromeric chromatin in human cells, D. melanogaster and yeast have demonstrated that the histone-fold domain is required for its centromere-specific deposition [Black et al., 2007].

In the fission yeast *S. pombe*, the genetics of CENH3 incorporation are relatively well defined. Here there are two separate pathways for CENH3 incorporation one operating at S phase and the other in G2. S-phase

incorporation requires a GATA family member, Ams2 [Takahashi et al., 2005] and the G2 pathway is dependent upon the *S. pombe* homologues of RbAp46 and RbAp48 [Hayashi et al., 2004; Takahashi et al., 2005]. These proteins have a number of functions in chromatin and are associated with chromatin-remodeling complexes, chromatin-modifying enzymes, and histone chaperones.

A recent study utilizing SNAP tag labeling to follow CENH3 through the cell cycle has helped clarify the timing and mechanism of CENH3 incorporation in human cells [Jansen et al., 2007]. The SNAP tag is a fusion tag consisting of the human O^6-alkylguanine transferase enzyme. Proteins tagged with this enzyme can be analyzed using an in vivo pulse-chase covalent labeling with cell-permeable fluorescent probe conjugated O^6-benzylguanine suicide substrates. The disappearance of the fluorescently tagged protein can then be examined over time. CenH3 bound at a mature centromere is unusually stable with little disassociation over the 50 h time frame of observation. These bound CENH3 were quantitatively and equally partitioned to sister centromeric DNA generated during S phase, and remained stably associated through multiple cell divisions. Surprisingly little new CENH3 was incorporated at S phase to replace those nucleosomes which segregate to opposite DNA daughter strands, instead normal H3 containing nucleosomes were incorporated. Addition of new CENH3 containing nucleosomes was restricted exclusively to the subsequent G1 phase. A mitosis intervening between centromere DNA replication and new CENH3 loading was found to be a prerequisite for CENH3 recruitment during G1. Earlier studies suggested that CENH3 may load in G2 phase based on an increase in overall CENH3 protein levels at this time [Shelby et al., 2000], however the SNAP tag study indicates that this CENH3 is nuclear but in a form free from the centromere. CENH3 protein was expressed throughout the cell cycle but only became centromere associated during the brief early G1 window.

A model based on these results is shown in Figure 2. Here the dilution of CENH3 to daughter DNA strands at S phase and the incorporation of the prevalent H3 containing nucleosomes at this time create the phased epigenetic state that promotes loop formation and orientation of CENH3 to initiate base plate formation and kinetochore assembly. The replacement of H3 nucleosomes with those containing CENH3 at G1 reinforces epigenetic marking of the centromere, while the subsequent dilution of this signal during S phase resets the epigenetic signature providing a marker for proper kinetochore assembly.

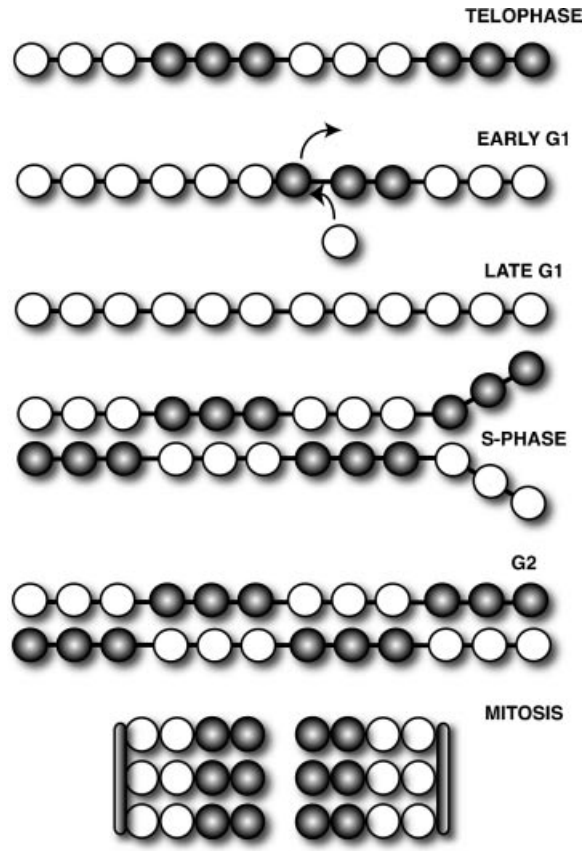

Fig. 2. Cell cycle-dependent changes in centromeric histone composition. At the onset of interphase, centromeric chromatin contains a mixture of histone H3-containing nucleosomes (dark circles) and CENH3-containing nucleosomes (white circles). During early G1, the histone H3-containing nucleosomes are replaced with CENH3 through a histone exchange process. As the DNA is replicated during S-phase, the CENH3-containing nucleosomes segregate to both daughter strands. New nucleosomes containing histone H3 are also assembled onto the replicating DNA resulting in a centromeres that contains a mixture of CENH3 and histone H3-containing nucleosomes in the centromeric domains of G2 chromatids. During mitosis, the chromatin folds three-dimensionally such that the CENH3-containing nucleosomes associate with the outer face of the chromosome to form the base of the kinetochore (bars on the left and right side of the mitotic chromosome).

One inconsistency in the literature is that over-expressed CENH3 loads into chromatin in a promiscuous manner preferentially in domains of euchromatin [Collins et al., 2004],

while CENH3 bound at the mature centromere appears to be extremely stable [Jansen et al., 2007]. One explanation is that CENH3 may bind relatively easily to euchromatin but without additional interactions this association may be short lived. In contrast insertion of CENH3 in place of euchromatin marked (dime K4 H3) H3 containing nucleosomes in centromeric chromatin may be stabilized by the pre-existing CEN chromatin conformation and perhaps further stabilized by constitutive centromere binding proteins. CENH3 incorporated in this environment may be additionally stabilized by the deposition of the CENP-A nucleosome-associated complex (CENP-ANAC) and subsequent downstream kinetochore components. It is tempting to speculate that the factors involved in loading CENH3 may do so in part by recognizing some aspect of the diMe K4 H3 marked chromatin. This raises the possibility that a portion of the H3 containing nucleosomes which dilute the CENH3 mark after S phase are modified with the dime K4 H3 euchromatin epigenetic marker specifically to insure that, when appropriate in G1, some of these residues are swapped for CENH3 containing nucleosomes by histone modifying complexes. Such a mechanism would amplify the intensity of the CENH3 epigenetic signal to a level similar to that found prior to dilution at S phase. This scenario would result in a self-renewing and self-reinforcing epigenetic state favorable to reliably mark centromere location as well as to provide the optimal chromatin configuration for kinetochore formation.

WHAT IS THE NATURE OF THE CENH3 LOADING FACTOR(S) ACTIVE AT G1?

Recently Maddox et al. identified a conserved Myb domain containing protein family necessary for CENH3 loading in *C. elegans*. The KNL-2 protein is a member of the M18BP1/hsKNL2 protein family identified previously by Fujita et al. as being important for CENH3 loading. This is a potentially exciting finding as the myb domain has the ability to bind short specific DNA sequences and thus has the potential to allow DNA sequence to direct CENH3 recruitment [Maddox et al., 2007]. The significance of this characteristic awaits experimental determination.

Two additional factors involved in CENH3 loading require mention. The first is the nonhistone protein Scm3 from budding yeast *S. cerevisiae* [Camahort et al., 2007; Mizuguchi et al., 2007; Stoler et al., 2007]. This protein is essential for recruitment of the CENH3 homolog Cse4 to centromeres and for segregation fidelity. Scm3 forms a complex with Cse4, H4 tetramers in centromeric chromatin, suggesting the presence of nucleosomes that lack H2A and H2B. Together, the studies suggest a hexamer nucleosome structure composed of two each of Scm3, Cse4, and histone H4 superficially resembling the hemisome isolated in Drosophilia [Dalal et al., 2007]. These two examples of non-octameric nucleosomes require a reinterpretation of the classical model. Scm3 is not conserved in higher eukaryotes, however similar nucleosome components have not been ruled out.

The latest factor described that affects CENH3 loading is Sim3, a protein isolated from fission yeast that is homologous to the human histone binding protein NASP. Cells defective in Sim3 function have reduced levels of CENH3 at centromeres and display chromosome segregation defects. Sim3 is thought to act as an escort for CENH3, handing it off to chromatin assembly factors that then incorporate it into centromeric chromatin [Dunleavy et al., 2007]. A global model of CENH3 loading requires discovery of all factors involved and further definition of recruitment pathways.

A STRUCTURAL ROLE FOR PERICENTRIC HETEROCHROMATIN IN TENSION SENSING AND SPINDLE ASSEMBLY CHECKPOINT INACTIVATION

A body of literature confirms that disruption of pericentric heterochromatin conformation leads to chromosome segregation defects [Melcher et al., 2000; Blower and Karpen, 2001; Nonaka et al., 2002; Fukagawa et al., 2004; Guenatri et al., 2004]. In most cases, the defects observed have been explained by the loss of cohesin accumulation associated with this domain. We have previously reported the dynamic nature of the epigenetic modification trimethylation of lysine 9 on histone H3 during the cell cycle. We found that mouse cells deficient in Suv(var)3-9, the enzyme responsible for this epigenetic mark, and cells whose dynamic methylation was experimentally blocked both displayed chromosome segregation defects [McManus et al., 2006]. In a

continuation of this study we have examined the effect of inhibiting histone methylation for brief periods during the cell cycle using the general methylation inhibitor, adenosine dialdehyde. We found that treated cells have significantly reduced levels of methylation on lysine 9 of histone H3 (H3K9me3) and lysine 20 of histone 4 (H4K20me3). A large proportion of cells treated in this manner accumulate at metaphase, with chromosomes loosely aligned on the metaphase plate compared with controls. These cells delay metaphase to anaphase transition suggesting activation of the spindle assembly checkpoint. Eventually the cells fail to divide resulting in the accumulation of large tetraploid cells with irregular nuclear boundaries showing that spindle checkpoint inactivation failed to occur. Examination of centromere and kinetochore structure of treated and control cells by transmission electron microscopy showed disruptions in the form and structure of the tri-laminar kinetochore plate. Centromeric chromatin appeared de-condensed and the strength of the association between the kinetochore and the underlying CEN chromatin appeared weakened. In samples under tension, the kinetochore appeared to separate from the centromere. An examination of inter-kinetochore distance confirmed that the kinetochores of treated cells were under reduced tension. These results indicate that intact pericentric heterochromatin is necessary for spindle assembly checkpoint inactivation (R. Heit, G. Chan, and M.J. Hendzel, manuscript in preparation). This can be at least partially explained by a requirement for fully condensed pericentric heterochromatin to properly sense the tension generated by sister kinetochores with proper bi-polar spindle attachment (Fig. 3).

The spindle checkpoint function of the kinetochore appears to sense both MT attachment and the tension this generates, delaying metaphase to anaphase transition until all sister kinetochores initiate proper bipolar spindle attachment. Cycles of MT polymerization and de-polymerization create oscillary motion that tests MT connections and eventually orders attached chromosomes on the mitotic plate. Once in position, the sister kinetochores come under more uniform tension, directing mechanical strain through the sister centromere kinetochore ultrastructures. It is this tension that seems to signal the correct conditions for high fidelity chromosome segregation, inactivating the checkpoint and allowing the Anaphase Promoting Complex (APC) to degrade cohesin, irreversibly initiating anaphase.

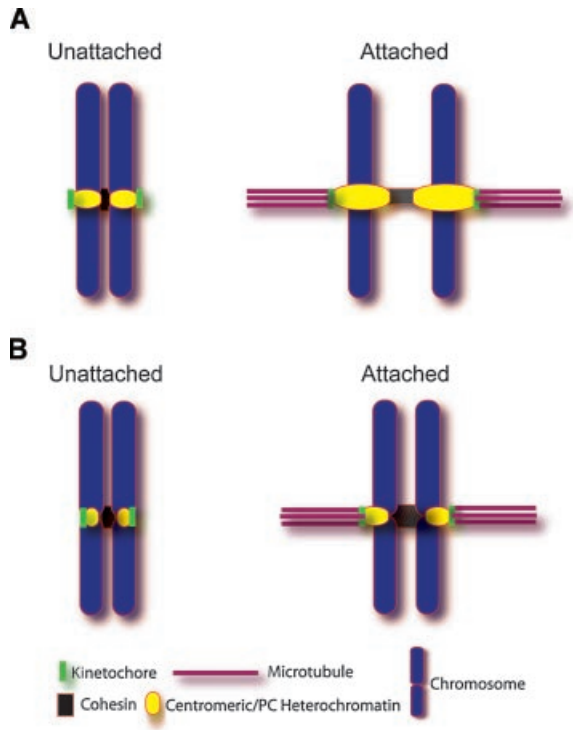

Fig. 3. The involvement of centromeric and pericentromeric heterochromatin in tension-sensing and mitotic checkpoint function. **A**: Cells with epigenetic defects in pericentromeric heterochromatin have a structurally compromised heterochromatin organization. When bipolar attachment is achieved, the heterochromatin is less able to resist the applied tension and may decondense in response to this tension. The consequence of this decondensation is that the tension requirement for spindle checkpoint inactivation may not be achieved because the tension is absorbed by the unfolding chromatin rather than the kinetochore itself. **B**: Under normal conditions, the centromeric and pericentromeric heterochromatin remain structurally stable under tension. This allows the force to be transmitted fully to the kinetochore and allows the spindle checkpoint to be satisfied.

Unattached kinetochores and MT attachments that do not generate tension activate the checkpoint. Syntelic (both kinetochores attached to MT from one pole) and merotelic (one sister kinetochore bound to MT from both poles, with its sister kinetochore attached only to one pole) attachments fail to deliver uniform tension and are destabilized in a process involving Aurora kinase B mediated phosphorylation of target proteins such as Ndc80 [Pinsky and Biggins, 2005]. The destabilization of MT attachment by Aurora B creates

unattached tensionless kinetochores, which simultaneously activate the spindle checkpoint.

The "wait" signal involves formation of the Mitotic Checkpoint complex (MCC), which contains the APC activator cdc20 and the spindle-assembly checkpoint proteins Mad2, Mad3, and Bub3. Mad2 is a unique two state molecule regulated through a complex set of interactions with factors such as Mad1 and p31comet [Yang et al., 2007]. Mad2 accumulates within the MCC at unattached kinetochores and interacts with cdc20 negatively regulating its ability to activate the APC complex. In the absence of APC activity cohesin remains intact and mitotic progression is halted [Musacchio and Salmon, 2007]. Although the exact mechano-chemical mechanisms involved in tension sensing remain elusive, the key components and some of their functions are known. Shugoshin has a role in loading Aurora B kinase at centromeres and is required to sense tension [Indjeian et al., 2005]. Polo like kinase 1 (Plk1) phosphorylates kinetochores that are not under tension. Once under uniform tension at the metaphase plate phosphorylation wanes, but this is immediately reversed if tension is ablated by the addition of MT de-stabilizing drugs. Plk1 appears to co-localize with an antibody (3F3/2) recognizing its Phosphoepitope at the mid-to-outer layers of the tri-laminar plate [Ahonen et al., 2005]. Plk1 interacting checkpoint helicase (PICH) binds Plk1 and also localizes to the inner tri-laminar plate where it integrates with centromeric chromatin [Baumann et al., 2007]. PICH is essential for accumulation of Mad2 at kinetochores in a tension dependent manner. In the most current model, cantenated CEN chromatin is thought to stretch in reaction to bipolar kinetochore tension causing PICH to lose the conformation or activity necessary for Mad2 accumulation within the MCC. Mad2 loss allows cdc20 to activate the APC complex leading to cohesin loss and anaphase initiation. As anaphase ensues, PICH coated chromatin fibers can be seen emanating from recently detached sister kinetochores suggesting that catenated sections of PICH coated CEN chromatin selectively stretch out in response to the intense strain of still incomplete chromatid separation [Baumann et al., 2007]. Whether this depends on chromatin remodeling by PICH remains to be determined. It takes the unique de-catenating activity of Topoisomerase II to resolve the PICH chromatin treads finally allowing independent progression of chromatids to the mitotic poles [Spence et al., 2007].

Our recent results are consistent with this model. The force created by bipolar mitotic spindle attachment passes through the mature tri-laminar plate of the centromere. One consequence of pericentric heterochromatin decondensation would be a reduced ability to transmit such force without this chromatin domain itself deforming in response. This force must be sensed mechanically by PICH to allow the biochemical cascade resulting in SAC inactivation and formation of the active Anaphase promoting complex. The ability of pericentric heterochromatin to transmit force is dependent on its full condensation. The epigenetic marks (H3K9me3) and (H4K20me3) are important in this function [Melcher et al., 2000; Schotta et al., 2004], indicating that chromatin modification is essential for the tri-laminar plate to act effectively as a structural element in tension sensing and SAC inactivation. The sensitivity of any tension sensing scenario would be dependent upon the mature structure withstanding strain and transmitting force to the center of the tri-laminar plate where the current model suggests stretching of PICH coated CEN chromatin may be the key tension sensing mechanism. Stretching in other areas where heterochromatin integrity is diminished would complicate spindle checkpoint inactivation and lead to the defective chromosome segregation found in experimental systems and disease conditions where pericentric chromatin condensation is compromised. From these studies we conclude that epigenetic modification both marks centromere location and dictates the functional conformation of the mature tri-laminar structure.

CONCLUSION

The debate regarding the influence of DNA sequence on centromere location is mooted by the fact that centromeres are reliably maintained, with minor shifting, generation after generation within these α-satellite stretches. Neocentromere formation is rare. De novo centromere formation on artificial human chromosomes also rarely occurs outside of repetitive α-satellite DNA. Thus it is apparent that centromeres evolved to prefer the confines of repetitive DNA. The key may lie in the fact that two identical repetitive domains of DNA can be

modified by epigenetic mechanisms to take on differing 3D conformations and thus functions, or the same two stretches can be similarly modified such that two even distal domains have similar conformation and function. This mechanism allows epigenetic modification to define function, whether this is liquid crystal like packaging, cohesin accumulation, or structural integrity.

The exact nature of the CENH3 nucleosome must be determined, especially in light of findings suggesting a configuration differing from the usual octamer. As well the pathway responsible for CENH3 loading must be more fully defined. While it is consensus that CENH3 incorporation defines centromeric chromatin and regulates kinetochore formation, there appears to be a role for each of the other distinguishing epigenetic marks to form a fully functional centromere/kinetochore ultrastructure (Table I). The phasing of CENH3 with H3 and diMe-K4 H3 required to mark centromere location and initiate kinetochore formation must also be more fully examined.

The role of pericentric heterochromatin should not be understated. The centromere of almost all higher eukaryotes is continuous with at least one domain of heterochromatin, and this is likely the case for functional neocentromeres as well. Its importance is further highlighted by the growing body of literature showing disruption of heterochromatin by many mechanisms results in chromosome segregation defects. The importance of this chromatin domain for tri-laminar plate formation and cohesin accumulation are well documented, however pericentric heterochromatin may also contribute structural strength to the centromere/kinetochore ultra-structure required for tension sensing and spindle checkpoint inactivation. Studies are currently underway to address this possibility. The severe centromeric defects seen in ICL patients with extreme hypomethylation of satellite and α-satellite DNA and de-condensation of heterochromatin also requires that this enzyme and its epigenetic mark be experimentally investigated.

We conclude that the full function of the mature centromere-kinetochore tri-laminar plate is dependent on its associated epigenetic modifications (Table I). Together these dictate the position and conformation of the centromere and allow the chromatin to provide the essential functions of kinetochore assembly, chromatid cohesion and tension sensing required for genetic stability. These studies further our understanding of how epigenetic modifications regulate such dynamic and critical cellular components. A full understanding of these variables will allow construction of stable artificial chromosomes useful in research and clinically, for gene replacement therapies. By fully understanding the mechanisms of successful chromosome segregation we may eventually target the aneuploidy often associated with cancer.

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