REVIEW / SYNTHÈSE

Epigenetic regulation of centromere formation and kinetochore function[1]

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Abstract: In the midst of an increasingly detailed understanding of the molecular basis of genome regulation, we still only vaguely understand the relationship between molecular biochemistry and the structure of the chromatin inside of cells. The centromere is a structurally and functionally unique region of each chromosome and provides an example in which the molecular understanding far exceeds the understanding of the structure and function relationships that emerge on the chromosomal scale. The centromere is located at the primary constriction of the chromosome. During entry into mitosis, the centromere specifies the assembly site of the kinetochore, the structure that binds to microtubules to enable transport of the chromosomes into daughter cells. The epigenetic contributions to the molecular organization and function of the centromere are reviewed in the context of structural mechanisms of chromatin function.

Key words: centromere, kinetochore, chromatin, CENP-A, intrinsic disorder, pericentromeric heterochromatin, histone H3 lysine 9 methylation.

Résumé : Malgré la compréhension de plus en plus détaillée que l'on a des bases moléculaires de la régulation génomique, on ne connaît encore que vaguement la relation qui existe entre la biochimie moléculaire et la structure de la chromatine à l'intérieur des cellules. Le centromère est une région structurellement et fonctionnellement unique à chaque chromosome qui fournit un exemple où la compréhension moléculaire dépasse de beaucoup la compréhension de la relation qui existe entre la structure et la fonction à l'échelle chromosomique. Le centromère est localisé à la constriction primaire du chromosome. Lors de l'entrée en mitose, le centromère spécifie le site d'assemblage du kinétochore, la structure qui se lie aux microtubules et permet le transport des chromosomes dans les cellules filles. La contribution épigénétique à l'organisation moléculaire et à la fonction du centromère est passée en revue dans le contexte des mécanismes structuraux de la fonction de la chromatine.

Mots clés : centromère, kinétochore, chromatine, CENP-A, disordre intrinsèque, hétérochromatine péricentromérique, histone H3 méthylation de lysine 9.

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The centromere

The centromere is essential for the correct segregation of sister chromatids during cell division. During entry into mitosis, the centromeric (CEN) chromatin specifies the initiation of the assembly of the kinetochore (Chan et al. 2005), which is a massive multiprotein assembly occupying a surface area of approximately 0.2 μm^2/kinetochore (Cherry et al. 1989). The kinetochore mediates microtubule attachment at the centromeres during mitosis. Remarkably, as CEN sequences have been characterized across species, the centromere was found to be one of the fastest evolving regions of the genome (Malik et al. 2002). Although this may account for the lack of primary sequence conservation of the centromere, certain elements, such as alphoid satellite DNA and GGAAT repeats, are common (Grady et al. 1992; Nakano et al. 2003) and are known to contribute to the assembly of the human centromere. For instance, the centromere protein (CENP) CENP-B is a DNA binding protein that binds specifically to a 17 bp sequence repeated in α-satellite DNA (Masumoto et al. 1989; Muro et al. 1992). Ectopic alphoid satellite sequences will preferentially recruit kinetochore proteins and form active centromeres when placed under selective pressure (Nakano et al. 2003).

Although the DNA binding specificity of CENP-B is consistent with a model in which DNA sequence dictates centromere formation, this can be ruled out because CENP-B is not required for the formation of a functional centromere (Kipling and Warburton 1997) and under normal conditions,

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alphoid DNA is not sufficient for the recruitment of many essential kinetochore proteins, such as CENP-A, CENP-C, and CENP-E (Nakano et al. 2003; Sullivan and Schwartz 1995; Warburton 2001; Warburton et al. 1997) (see the section of this review entitled The biochemistry of chromatin at the centromere). The migration of centromeres within otherwise conserved arrangements of genes and the existence of neocentromeres in humans provide convincing evidence that centromere specification is determined by an epigenetic rather than a sequence-specific mechanism (Warburton 2001).

Warburton (2001) did a comprehensive cataloguing of protein recruitment to normal, neo-, and inactive centromeres. Neocentromeres are fully functional centromeres that do not contain α-satellite DNA, while the term inactive refers to the inactive centromere in a dicentric chromosome. Certain characteristics, such as intense 4′,6-diamidino-2-phenylindole (DAPI) staining (denoting highly condensed AT-rich DNA) and α-satellite DNA, were shared among the normal and inactive centromeres but were absent from neocentromeres. Since neocentromeres are fully functional centromeres, yet lack α-satellite DNA, it can be concluded that these sequences are neither sufficient nor required for the formation of a centromere. This result was corroborated by a recent study showing that chromatin containing CENP-A is able to spread over non-α-satellite DNA when non-α-satellite DNA and α-satellite DNA are used to construct human artificial chromosomes (Lam et al. 2006). Thus, it is the composition of the chromatin, and not the underlying DNA sequence, which is important for specifying a functional centromere.

Chromatin structure and function

Epigenetic mechanisms are mediated through chromatin structure. This relationship has been evident since the earliest light microscopy studies on chromosomes and nuclei. The DNA present in the eukaryotic cell has been characterized by early light microscopists as comprising both euchromatin and heterochromatin. Heterochromatin is defined as the chromatin that remains compact and visible throughout the cell cycle. In contrast, euchromatin decondenses in interphase to the extent that it is no longer directly visible in the light microscope. Heterochromatin can be further categorized as constitutive or facultative, based upon whether or not there is a consistent relationship between the DNA sequence involved and a compact organization across cell types and differentiation states. The former is exemplified by CEN and pericentromeric heterochromatin, which are virtually always highly condensed, while the latter contains sequences that are either heterochromatic or euchromatic, depending on the cell type and state of differentiation. The inactivation of 1 of the 2 human X chromosomes in females is an example of facultative heterochromatin.

Beginning in the 1980s, biochemical properties that distinguish these different morphological classes of interphase chromatin have been identified and a mechanistic understanding of how heterochromatin is established and maintained began to emerge. In this regard, DNase I digestion kinetics have proven to be one of the most revealing features of chromatin and reflect the close relationship between structure and function, where differences in function are reflected in the accessibility of the underlying DNA sequence to the nuclease probe. At the coarsest level, sequences that are transcribed or in a chromatin conformation compatible with transcription are digested about 3 times more rapidly than sequences that are never transcribed (Weintraub and Groudine 1976). Increased rates of digestion are correlated with an enrichment in histone acetylation and reduced histone H1 content (Iovcheva et al. 1984; Krajewski and Becker 1998; Perry and Annunziato 1989; Ridsdale et al. 1988). At higher resolution, small regions of sequences involved in transcriptional regulation are digested at rates that are at least 10 times faster than the surrounding sequences. These sites are consistently associated with the binding of proteins directly involved in the regulation of transcriptional activation (Keene et al. 1981; Lu and Richardson 2004).

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 (Luger et al. 1997). The linking DNA is associated with a fifth histone, histone H1, which binds DNA as it enters and exits the nucleosome to stabilize 2 complete turns of the DNA around the histone octamer (Thomas 1999). Histone proteins are the substrate for post-translational modifications. It is these post-translational modifications, largely located in the N-terminal domains of the histone proteins, which encode most of the epigenetic information specifying chromatin structure and function (Bradbury 1992; Davie 1996; Shilatifard 2006). For example, reduced acetylation of the N-terminal lysines of histone H4 (Braunstein et al. 1993; Kristjuhan et al. 2003; Richards and Elgin 2002), trimethylation of lysine 20 on histone H4 (Biron et al. 2004; Kourmouli et al. 2004; Schotta et al. 2004), trimethylation of lysine 9 on histone H3 (Fischle et al. 2003; Gonzalo et al. 2005; Peters et al. 2001; Rice et al. 2003), and trimethylation of lysine 27 on histone H3 (Chadwick and Willard 2004) have all been correlated with heterochromatin structures. The N-terminal domains of the histones are not required for the formation of the nucleosome (Hayes et al. 1991), but do regulate the folding of the nucleosome polymer into more compact conformations (Fletcher and Hansen 1995; Garcia-Ramirez et al. 1992; Krajewski and Ausio 1996).

The biochemistry of chromatin at the centromere

Chromatin in the centromere differs biochemically from the remainder of the genome in some very fundamental ways. Early studies indicated that 3 proteins, CENP-A, CENP-B, and CENP-C are specific to functional centromeres (Earnshaw and Migeon 1985). Following this discovery, sequencing of CENP-A revealed it to be a homolog of histone H3 (Palmer et al. 1987, 1989, 1991). These results were corroborated by the discovery that CENP-A substitutes for H3 in active CEN and neocentromeric nucleosomes, but

is not present at inactive centromeres (Palmer et al. 1987; Warburton 2001; Warburton et al. 1997). In addition to the obvious functional differences between CEN and nonCEN chromatin, CEN heterochromatin is much less effective than the surrounding pericentromeric heterochromatin at repressing transcription (Allshire et al. 1994; Lam et al. 2006; Pidoux and Allshire 2005).

An important advance in our understanding of the epigenetic status of CEN heterochromatin came with the analysis of linearized chromatin fibers. By stretching chromatin on glass slides, Sullivan and Karpen (2004) mapped histone H3 and CENP-A distribution in HeLa and *Drosophila* CEN chromatin. This study determined that the *Drosophila* CENP-A homologue CID is incorporated as 10–40 kb long clusters of nucleosomes interspersed with H3-containing nucleosomes. Sullivan and Karpen (2004) were also able to define the post-translational modification status of CEN heterochromatin. They showed that the H3-containing nucleosomes within CEN chromatin were hypoacetylated, which is typical of heterochromatin, and enriched in dimethylated lysine 4, a modification typically associated with potentiated regions of chromatin (see also Lam et al. 2006). This result has been validated by chromatin immunoprecipitation experiments (Cam et al. 2005; Lam et al. 2006).

Using a human artificial chromosome model, Lam et al. (2006) used this same linearization of chromatin technique, combined with chromatin immunoprecipitation, to show that CEN and pericentromeric heterochromatin exist in a dynamic equilibrium. Under normal conditions, CEN heterochromatin that contains CENP-A is flanked by chromatin enriched in dimethylated lysine 9, which separates the CEN heterochromatin from the pericentromeric constitutive heterochromatin. Constitutive heterochromatin is demarked by enrichment in trimethylated lysine 9. When CENP-A is overexpressed, the CEN heterochromatin expands and displaces dimethylated lysine 9-enriched chromatin. The trimethylation of lysine 9 in the flanking pericentromeric heterochromatin also increases, which Lam et al. (2006) suggest is a compensatory mechanism in response to expanding CEN chromatin.

The centromere-specific histone H3 homologue CENP-A

Collectively, these results imply that CENP-A is the critical feature that specifies the formation of a functional centromere. CENP-A association, however, is insufficient for centromere formation. This has been shown by the incorporation of CENP-A into regions of the genome outside of centromeres as a result of CENP-A overexpression. These experiments have demonstrated that CENP-A is not sufficient to generate a functional centromere (Van Hooser et al. 2001). Nonetheless, it is equally clear that CENP-A incorporation is a feature of all functional centromeres.

The greatest divergence in CENP-A and histone H3 is in the N-terminal sequence. Interestingly, the N-terminus of CENP-A also differs significantly among species (Fig. 1). It has been suggested that this divergence of the N-terminal tail of CENP-A homologs reflects the divergence in the underlying DNA sequences that function as centromeres among different species (Malik et al. 2002) . In this context, the N-terminal domain of CENP-A is thought to interact with linker DNA connecting individual nucleosomes. Moreover, in *Saccharomyces cerevisiae,* the CENP-A homolog requires the N-terminal tail for proper function. CENP-A is required for the assembly of at least 1 protein complex at the yeast centromere (Chen et al. 2000), and is able to recruit CENP-C, hSMC1, and hZW10 in human cells under conditions in which functional centromeres are not formed (Van Hooser et al. 2001).

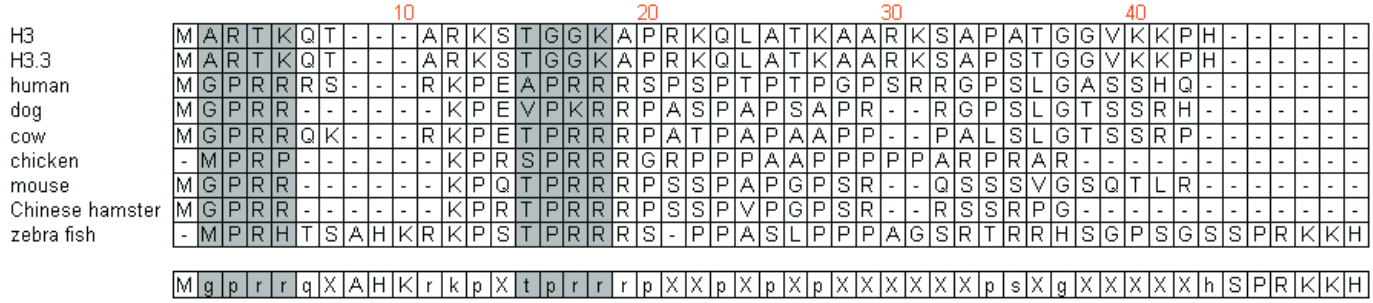
It is noteworthy that the N-terminal domain of CENP-A proteins contain a sequence repeat that is similar to the SPKK DNA binding motif found in the C-terminal domain of H1 histones (Churchill and Suzuki 1989; Malik et al. 2002). (Fig. 1). The N-terminal domain of CENP-A may be similar to the C-terminus of histone H1 in another important way. The C-terminus of histone H1 is unstructured in solution but is now thought to adopt secondary and tertiary structure upon binding to DNA at the surface of the nucleosome (Hendzel et al. 2004; Roque et al. 2005; Vila et al. 2000, 2001). The C-terminus is also engaged in a number of different but functionally important interactions within chromatin (Hansen et al. 2006). The ability of a discrete polypeptide sequence to engage in multiple specific interactions is a property that is common to proteins with unusually high proportions of disorder-producing amino acids (see Table 1 for the composition of CENP-A). Such proteins can adopt remarkably different structures for the same polypeptide sequence, thereby allowing very different yet specific protein–protein interactions (Dunker et al. 2005). We therefore propose that the CENP-A N-terminal domain, similar to what has been suggested for the histone tail domains (Hansen et al. 2006), is intrinsically disordered and adopts unique secondary and tertiary structures specific to the interacting ligand. This would provide versatility to the domain despite being coded for by a short stretch of sequence.

Although the N-terminal domain represents the site of greatest divergence with the major histone H3 subtypes, the N-terminal domain does not specify CENP-A incorporation into the centromere. Rather, the changes that dictate incorporation specifically into the centromere are contained within the globular domain of histone H3. Divergence from histone H3 in the L1 loop and the α2-helix are necessary and sufficient to target CENP-A to the centromere (Black et al. 2004). When this domain is placed into the histone H3 sequence, the synthetic histone H3 targets to the centromere (Black et al. 2004). Using deuterium exchange coupled with mass spectrometry, Black et al. (2004) further demonstrated that the CENP-A/H4 tetramer is more compact and structurally rigid than the H3/H4 tetramer. Although speculative, an altered nucleosome structure may contribute to the loss of micrococcal nuclease nucleosomal repeat pattern that has been observed at centromeres (Saitoh et al. 1997). This altered micrococcal nuclease digestion pattern has been shown to require CENP-A incorporation (Takahashi et al. 2000).

The assembly of CENP-A into centromeric chromatin

Since CENP-A replaces histone H3 in nucleosomes within CEN chromatin, it might be expected that CENP-A incorporation occurs during S phase. It has long been known that

Fig. 1. Alignment of the N-terminal domain of vertebrate CENP-A. The N-terminal sequences from human histone H3.2 (H3) and H3.3 are shown in comparison with CENP-A from several vertebrates. The regions highlighted in grey indicate the positions of motifs that may be homologous to the SPKK (serine-proline-lysine-lysine)-type repeats found in the C-terminal domain of H1 histones.



euchromatin and heterochromatin replicate at different points during S phase, and this could be a mechanism by which uncommon histone variants are incorporated. By examining the replication timing of several chromosomes in *Drosophila*, the timing of centromere replication was found to vary among chromosomes from early to late S phase (Sullivan and Karpen 2001). The replication of H3-containing nucleosomes and CID-containing nucleosomes, both within the centromere, were also shown to occur at the same time (Blower et al. 2002). Similar results were observed in mammalian systems. When dicentric CHO cells were examined, CENP-A was incorporated into active, but not inactive, centromeres. Both the active and inactive centromeres were then compared, and no difference in replication timing was found (Ouspenski et al. 2003). Similarly, HeLa cells were shown to replicate CENP-A-associated sequences in mid- to late-S phase, overlapping with the replication timing of other regions in the chromosome (Blower et al. 2002; Shelby et al. 2000; Sullivan and Karpen 2001).

Although histone proteins incorporated into the nucleosome are among the most stable proteins in the nucleus, it is also well established that histones can be incorporated into chromatin outside of S-phase (Kimura 2005). CENP-A is able to be incorporated into CEN DNA in the presence of aphidicolin, a DNA replication inhibitor (Shelby et al. 2000), indicating that CENP-A is similarly capable of incorporation into chromatin outside of S phase. This implies that replication-independent nucleosome assembly is the mechanism of specifying CENP-A incorporation into the centromere. Consistent with this hypothesis, the analysis of CENP-A gene expression in HeLa cells revealed that CENP-A mRNA and protein expression were found to be most abundant in G2 (Shelby et al. 2000). When CENP-A expression is forced throughout S phase, its incorporation is not centromere specific (Shelby et al. 1997). Thus, replication-independent nucleosome assembly may be essential to maintain the specificity of CENP-A incorporation.

Replication-independent nucleosome assembly

The H3/H4 tetramer, which forms the core of the nucleosome, is vastly more stable in vivo than most protein–DNA complexes (Kimura and Cook 2001), which turn over in seconds to minutes (Phair et al. 2004). Despite this, there is a small amount of incorporation of new H3 and H4 outside of S phase (Hendzel and Davie 1990; Jackson 1990). During G1 and G2, a unique histone H3 variant, histone H3.3, is incorporated into chromatin and serves as an example of replication-independent nucleosome assembly (Ahmad and Henikoff 2002; Hendzel and Davie 1990). This is accomplished through a chromatin assembly factor, HIRA, which specifically recognizes histone H3.3 (Ray-Gallet et al. 2002; Tagami et al. 2004). The existing data support the hypothesis that CENP-A, like histone H3.3, is incorporated into nucleosomes in G2 (Shelby et al. 1997, 2000). It is therefore expected that a unique chromatin assembly factor that is specific for CENP-A and responsible for the deposition of CENP-A specifically at centromeres will be identified. The CENP-A protein itself has been postulated to serve as the epigenetic mark that directs CENP-A incorporation after S phase. The equal segregation of nucleosomes that contain CENP-A to daughter strands would result in the presence of a CENP-A mark interspersed in H3.2 and (or) H3.1. Alternatively, other epigenetic marks that are reported to be present at centromeres, such as unacetylated histone H3 that is dimethylated at lysine 4, could provide the target for chromatin remodeling machinery and the incorporation of additional CENP-A into chromatin by a unique chromatin assembly factor.

In *Schizosaccharomyces pombe*, the genetics of CENP-A incorporation are relatively well defined. In this organism, there are 2 separate pathways for CENP-A incorporation and the protein is incorporated in both S phase and in G2. S-phase incorporation requires a GATA family member, Ams2 (Takahashi et al. 2005). The G2 pathway, however, is of particular relevance to the replication-independent assembly that appears to operate in mammalian cells. This pathway is dependent upon the *Schizosaccharomyces 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 (Loyola and Almouzni 2004; Zhang et al. 1999). In *Schizosaccharomyces pombe*, these proteins are responsible for recruiting a complex of Mis6, Mis 15, and Mis 17 (Hayashi et al. 2004). Depletion of RbAp46 and RbAp48, but not either one alone, was sufficient to deplete CENP-A from the centromeres of HeLa cells and led to defects in chromosome segregation (Hayashi et al. 2004) Thus, a complex containing both proteins or complexes involving RbAp46 and (or) RbAp48 appear to specify a replication-independent pathway for CENP-A assembly that is conserved from *Schizosaccharomyces pombe* to mammals.

Constitutive and cell-cycle dependent proteins associated with the centromere

In addition to CENP-A, other proteins have been found to localize to the centromere throughout the cell cycle. Since most kinetochore proteins are recruited to the centromere only at mitosis, proteins that are constitutively present at the centromere may play an important role in the specification of the centromere. To date, it is evident that at least 6 proteins are constitutively centromeric, CENP-A, CENP-B, CENP-C, CENP-H, CENP-I (hMis6), and hMis12 (Chan et al. 2005), and thus each could function within the specification of the centromere. As mentioned previously, CENP-B is not required for a functional centromere (Perez-Castro et al. 1998; Warburton 2001) and so must not be a crucial protein for centromere formation. Recruitment of each of CENP-C, CENP-H, and CENP-I appear to be downstream of CENP-A localization. CEN localization of CENP-C requires both CENP-H and CENP-I, while CENP-H and CENP-I are dependant on each other for proper localization (Fukagawa et al. 2001; Nishihashi et al. 2002). Additionally, although CENP-A and hMis12 are both needed for CENP-I localization, the knockdown of any one of CENP-C, CENP-H, or CENP-I has no effect on CENP-A localization (Fukagawa et al. 2001; Goshima et al. 2003; Liu et al. 2003; Nishihashi et al. 2002). This contradicts the expectation, based on *Schizosaccharomyces pombe* experiments, that CENP-I, the human Mis12 homologue, is required for replication independent assembly of CENP-A into CEN nucleosomes (Hayashi et al. 2004). However, a more recent study indicates that a complex containing CENP-H and CENP-I, when depleted from DT40 cells, results in a failure to incorporate newly synthesized CENP-A (Okada et al. 2006). In all cases, however, these studies demonstrate that CENP-A incorporation is central to the assembly of a functional centromere.

Epigenetic regulation of the centromere in fission yeast

Schizosaccharomyces pombe has proven to be a very informative genetic model system for the study of centromere function and the epigenetic regulation of the centromere. The fission yeast system has clearly demonstrated the importance of pericentromeric heterochromatin in the function of the centromere.

In fission yeast, the pericentromeric heterochromatin is initiated through at least 2 independent pathways, the RNA interference (RNAi) pathway and a clr3 pathway (Hansen et al. 2005; Yamada et al. 2005). Clr3 is a fission yeast homologue of mammalian class II histone deacetylases (HDACs) and is required for the efficient deacetylation of lysine 14 of histone H3 in fission yeast centromeres (Bjerling et al. 2002; Wiren et al. 2005). Either pathway is capable of specifying the recruitment of clr4 (Nakayama et al. 2001), which is the fission yeast homologue of human SUV39H1 and H2, 2 enzymes responsible for the trimethylation of lysine 9 on histone H3 (Nakayama et al. 2001). Clr3 and clr4 were originally identified as genes required for the stable repression of the silent mating type loci in *Schizosaccharomyces pombe* and are part of a complementation group that includes swi6 (Ekwall and Ruusala 1994). The trimethylated form of histone H3 lysine 9 generates a binding site that is recognized by the chromodomain of swi6 (Hall et al. 2002). Swi6 is the fission yeast homologue of heterochromatin protein 1 (HP1) (Lorentz et al. 1994), both of which contain a chromodomain that binds to histone H3 trimethylated on lysine 9 (Hall et al. 2002). Mutations in the histone H3 N-terminus at lysine 9 or 14, as well as at serine 10, are sufficient to delocalize swi6 and result in defects in chromosome segregation (Mellone et al. 2003).

Unlike the *Schizosaccharomyces pombe* proteins associated with the centromere, those associated with the pericentromeric heterochromatin are not essential for viability. Loss of genes involved in the dicer (Provost et al. 2002; Volpe et al. 2003) and clr3 pathways of heterochromatinization (Grewal et al. 1998; Nakayama et al. 2001; Yamada et al. 2005) consistently generate a mitotic defect characterized by lagging chromosomes in anaphase. Both pathways converge on the clr4 histone methyltransferase, which establishes the swi6 binding site (Yamada et al. 2005). The swi6 protein has been shown to be directly involved in the recruitment of the cohesin complex, which is enriched at the inner centromere (Bernard and Allshire 2002; Bernard et al. 2001; Nonaka et al. 2002). When cohesin is disrupted, a similar phenotype is observed (Toyoda et al. 2002). Additional cohesion may be necessary to resist the force that is being directly applied to the kinetochore and to mechanically couple the chromatids.

During mitosis, the inner centromere binding protein (INCENP) targets the Aurora B kinase to the pericentromeric heterochromatin of the inner centromere (Adams et al. 2001*a*). Aurora B phosphorylates histone H3 at serine 10 at the onset of entry into mitosis. This correlates with the displacement of HP1 in both *Schizosaccharomyces pombe* and human cells (Fischle et al. 2005; Hirota et al. 2005). The purpose or function of HP1 displacement during mitosis is not known. Site-directed mutagenesis of lysine 9, serine 10, and lysine 14 revealed that each of these amino acids were important in recruiting HP1 to pericentromeric heterochromatin in *Schizosaccharomyces pombe* (Mellone et al. 2003). Moreover, mutations at each of these sites lead to chromosome segregation defects. The targeting of cohesin to pericentromeric heterochromatin has not been determined in these mutant backgrounds, but the requirement for HP1 in the assembly of cohesin into pericentromeric heterochromatin (Nonaka et al. 2002) predicts that cohesin assembly should also be impaired.

There may be contributions of protein methylation in kinetochore function that are independent of histones. In *Saccharomyces cerevisiae*, a histone methyltransferase, Set1, and an Aurora kinase, IpI1, have been shown to have similar antagonizing effects independent of histone modification (Zhang et al. 2005). The common substrate that seems to mediate this effect in *Saccharomyces cerevisiae* is a kinetochore protein, Dam1. Dam1 forms a collar around the end of microtubules (Westermann et al. 2005) and may couple microtubule depolymerization with chromosome movement by interacting with the kinetochore-associated Ndc80 complex (Salmon 2005). Although it is expected that a homologue of Dam1 exists in higher eukaryotes, none has been found to date. In addition, Set1p catalyzes the dimethylation of lysine 4 on histone H3, and mutation of this lysine to arginine partially suppresses the effects of loss of Ipl1 kinase function in

Table 1. Amino acid composition of the N-terminus of CENP-A.

			Amino acid							
			K	P	G	R	N	Q	S	E
Protein	Organism	Length	D	D	D	D	D	D	D	D
hCENP-A	*Homo sapiens* (human)	38	2.6	23.7	10.5	23.7	0	2.6	18.4	2.6
mCENP-A	*Mus musculus* (mouse)	33	3	21.2	9.1	21.2	0	9.1	21.2	0
rnCENP-A	*Rattus norvegicus* (rat)	57	1.8	22.8	10.5	21.1	0	5.3	17.5	0
btCENP-A	*Bos taurus* (cow)	36	5.6	27.8	5.6	19.4	0	2.8	8.3	2.8
cfCENP-A	*Canis familiaris* (dog)	33	6.1	24.2	9.1	21.2	0	0	15.2	3
cgCENP-A	*Cricetulus griseus* (hamster)	27	3.7	29.6	7.4	33.3	0	0	18.5	0
ggCENP-A	*Gallus gallus* (chicken)	29	3.4	44.8	3.4	31	0	0	3.4	0
drCENP-A	*Danio renio* (zebrafish)	45	8.9	22.2	6.7	20	0	0	20	0
dmCID	*Drosophila melanogastor*	116	2.6	8.6	4.3	14.7	6	7.8	11.2	6.9
dsCID	*Drosophila simulans*	115	2.6	8.7	5.2	13.9	6.1	7	13	7.8
dmaCID	*Drosophila mauritiana*	115	2.6	8.7	5.2	13.9	6.1	7	13	7.8
dseCID	*Drosophila sechellia*	115	2.6	8.7	5.2	14.8	7	7	12.2	7.8
dtCID	*Drosophila teissieri*	118	3.4	9.3	3.4	12.7	5.1	6.8	11	7.6
ceHcp-3	*Caenorhabditis elegans*	173	4.6	3.5	6.4	9.2	7.5	4	8.7	6.9
cecpar1	*Caenorhabditis elegans*	146	1.4	3.4	8.2	8.9	8.2	4.1	11	4.8
cbCENP-A	*Caenorhabditis briggsae*	158	8.2	5.1	1.9	10.1	5.7	3.8	7	13.9
spCENP-A	*Schizosaccharomyces pombe*	24	16.7	20.8	8.3	16.7	0	0	4.2	4.2
Cse4	*Saccharomyces cerevisae*	112	2.7	2.7	2.7	11.6	5.4	6.3	13.4	12.5
H3.3	*Homo sapiens*	37	21.6	5.4	10.8	10.8	0	5.4	8.1	0
H3	*Homo sapiens*	38	21.1	7.9	10.5	10.5	0	5.3	5.3	0
Globular H3	*Homo sapiens*	98	5.1	4.1	3.1	14.3	1	6.1	3.1	7.1

Note: D, disorder; N, order; O, neither; pI, isoelectric point.

Saccharomyces cerevisiae (although not as completely as the loss of Set1 function) (Zhang et al. 2005). Thus, in higher eukaryotes, the function of lysine methylation, and even of specific lysine methyltransferases, in kinetochore function may be complex and involve both histone and non-histone proteins.

Epigenetic regulation of centromere function and genomic stability in human cells

Human centromeres, like those of *Schizosaccharomyces pombe*, are also associated with pericentromeric heterochromatin domains that recruit HP1 through a mechanism that requires the methylation of histone H3 at lysine 9 (Fischle et al. 2005). Thus, we might expect that deletions of Suv39, the mammalian homologue of clr4, would compromise the function of the centromere. Knockout studies of the Suv39h1 and Suv39h2 homologs in mouse results in widespread genomic instability and increased incidence of lymphomas (Peters et al. 2001). However, the reported segregation defects, which are more consistent with increased cohesion and an impaired ability to dissociate cohesins, are not what would be predicted if the results from *Schizosaccharomyces pombe* directly applied to humans. More recently, we characterized chromosome segregation defects in immortalized mouse embryonic fibroblasts isolated from Suv39h1/h2 double null mice. We found an approximately 4-fold increase in the types of chromosome alignment and chromosome segregation defects observed in *Schizosaccharomyces pombe* (McManus et al. 2006). These same defects are observed in wild-type cells following as little as 2 h of treatment with a protein methylation inhibitor (Fig. 2). These results are consistent with a requirement for histone lysine methylation in the recruitment of cohesin to pericentromeric heterochromatin.

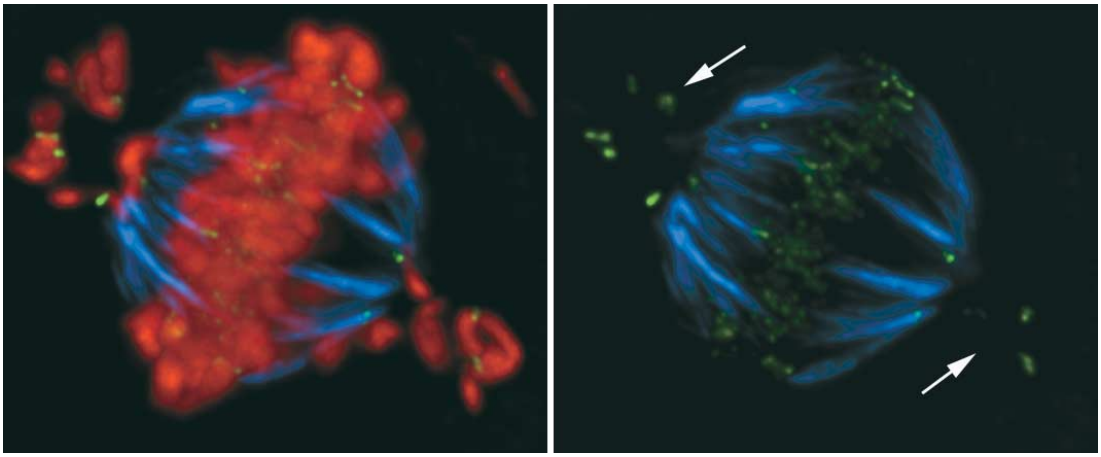
HDAC inhibitors are being developed as potential chemotherapeutic agents for the treatment of a spectrum of human cancers. Based upon the important roles of clr3 and clr6 HDACs in *Schizosaccharomyces pombe,* which deacetylate lysine 14 and lysine 9 of histone H3 (Bjerling et al. 2002), we would expect that inhibition of these proteins would cause segregation defects due to acetylation-dependent inhibition of lysine 9 trimethylation. This would be expected to result in a loss of cohesins at the inner centromere. HDAC inhibitors have been found to alter the formation of kinetochores on CEN DNA and cause a failure to properly capture microtubules and an accumulation of cells at prometaphase (Shin et al. 2003). In our hands, however, there are qualitative differences between the mitotic defects observed in the absence of trimethylation of lysine 9 and those seen with deacetylase inhibitor treatments (G.K. Chan and M.J. Hendzel, unpublished observations). Thus, it is not entirely clear that the mitotic defects observed with histone deacetylase inhibitors can be attributed to reduced recruitment of cohesin.

The function of pericentromeric heterochromatin in genomic stability and centromere function

Although the basic mechanisms of heterochromatin assembly are conserved in mammals and *Schizosaccharomyces pombe*, mammalian systems have additional machinery specifying the initiation of heterochromatin formation. In

D	M	A	T	V	H	F	I	L	C	W	Y			
D	D	N	N	O	O	O	O	O	O	O	O	D	O	pI
0	0	5.3	5.3	0	2.6	0	0	2.6	0	0	0	84.1	5.2	13.02
0	0	3	6.1	3	0	0	0	3	0	0	0	84.8	6	13.26
1.8	0	7	7	3.5	0	0	0	1.8	0	0	0	80.8	5.3	13.18
0	0	13.9	8.3	0	0	0	0	5.6	0	0	0	72.3	5.6	12.88
0	0	9.1	3	3	3	0	0	3	0	0	0	78.8	9	12.87
0	0	0	3.7	3.7	0	0	0	0	0	0	0	92.5	3.7	13.38
0	0	13.8	0	0	0	0	0	0	0	0	0	86	0	13.38
0	0	6.7	6.7	0	6.7	0	0	2.2	0	0	0	77.8	8.9	13.38
7.8	0.9	9.5	10.3	1.7	0.9	1.7	0	3.4	0	0	1.7	70.8	9.4	9.73
6.1	0.9	9.6	8.7	1.7	2.6	1.7	0	3.5	0	0	0.9	71.3	10.4	9.86
6.1	0.9	8.7	8.7	1.7	2.6	1.7	0	3.5	0.9	0	0.9	71.3	11.3	9.86
6.1	0.9	8.7	8.7	2.6	1.7	1.7	0	3.5	0	0	0.9	72.3	10.4	10.3
7.6	2.5	9.3	10.2	1.7	3.4	1.7	0	2.5	0.8	0	0.8	69.4	10.9	8.54
9.8	2.9	6.9	5.8	4.6	4.6	1.2	4.6	4.6	0	0	4	63.5	23.6	5.88
10.3	1.4	8.2	6.2	5.5	4.8	1.4	5.5	4.8	0	0	2.1	61.7	24.1	5.29
6.3	1.9	3.2	3.8	2.5	5.7	3.8	7.6	5.1	0.6	0.6	3.2	63.9	29.1	6.34
4.2	4.2	8.3	0	0	0	0	4.2	4.2	0	0	4.2	79.3	12.6	11.23
7.1	0.9	7.1	5.4	4.5	0.9	0.9	3.6	8	0	0.9	3.6	65.3	22.4	4.66
0	0	18.9	13.5	2.7	0	0	0	2.7	0	0	0	62.1	5.4	12.96
0	0	21.1	13.2	2.6	0	0	0	2.6	0	0	0	60.6	5.2	13
4.1	2	10.2	6.1	5.1	2	4.1	7.1	11.2	2	0	2	50	33.5	11.03

Fig. 2. Treatment with a protein methylation inhibitor induces chromosome alignment defects. Three-dimensional maximum point projection deconvolution images are shown of a HeLa cell treated for 2 h with adenosine dialdehyde prior to fixation. Microtubules are shown in blue, chromosomes are shown in blue, and anticentromeric antigen staining is shown in green.



mammals, this machinery includes the following: pericentromeric heterochromatin that is characteristically enriched in HP1 (Minc et al. 1999), trimethylation of lysine 9 on histone H3 (Peters et al. 2003), the trimethylation of histone H4 at lysine 20 (Biron et al. 2004; Schotta et al. 2004), hypoacetylated histones H3 and H4 (Johnson et al. 1998), and cytosine methylation of DNA (Henikoff 2000). The trimethylation of histone H4 at lysine 20 in heterochromatin is not conserved in *Schizosaccharomyces pombe* (Sanders et al. 2004). The enzymatic machinery that carries out these modifications is regulated by the Rb family of proteins and includes HDACs, histone methyltransferases, HP1, and DNA methylation (Brehm et al. 1998; Luo et al. 1998; Magnaghi-Jaulin et al. 1998; Pradhan and Kim 2002; Robertson et al. 2000; Vaute et al. 2002; Zhang et al. 2000). Not surprisingly, each of these pathways is linked to genomic instability and is associated with human cancers. The basis of this genomic instability, however, is not clear.

The study of mouse embryonic fibroblasts from triple-null mice for the 3 Rb family member genes revealed that the trimethylation of lysine 20 on histone H4, but not lysine 9 on histone H3, was lost (Gonzalo et al. 2005). Unfortunately, the analysis of the mitotic defects in these cells was restricted to karyotype analysis and ploidy determination.

Fig. 3. Cell-cycle associated changes in lysine 9 trimethylation. The neural tube of a 9.5-day-old mouse embryo stained with antitrimethylated lysine 9 (histone H3) and 4′,6-diamidino-2-phenylindole (DNA). A portion of the neural tube is shown. The cells nearest the lumen (left) are in various stages of mitosis, whereas the cells distant from the lumen are in interphase. The methylation is largely confined to the pericentromeric heterochromatin.

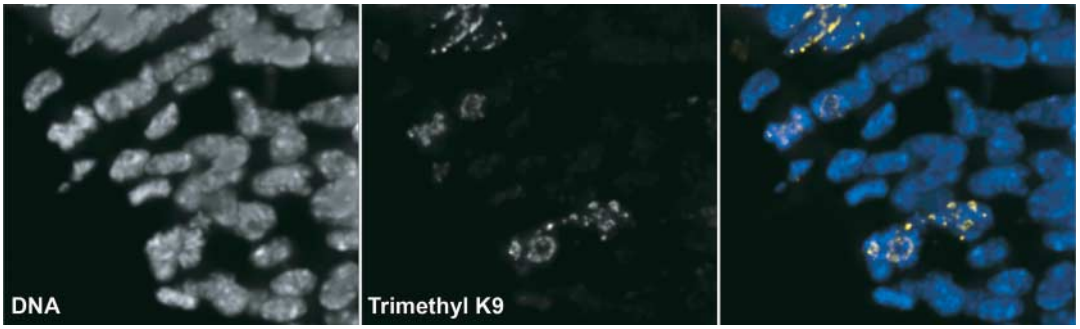
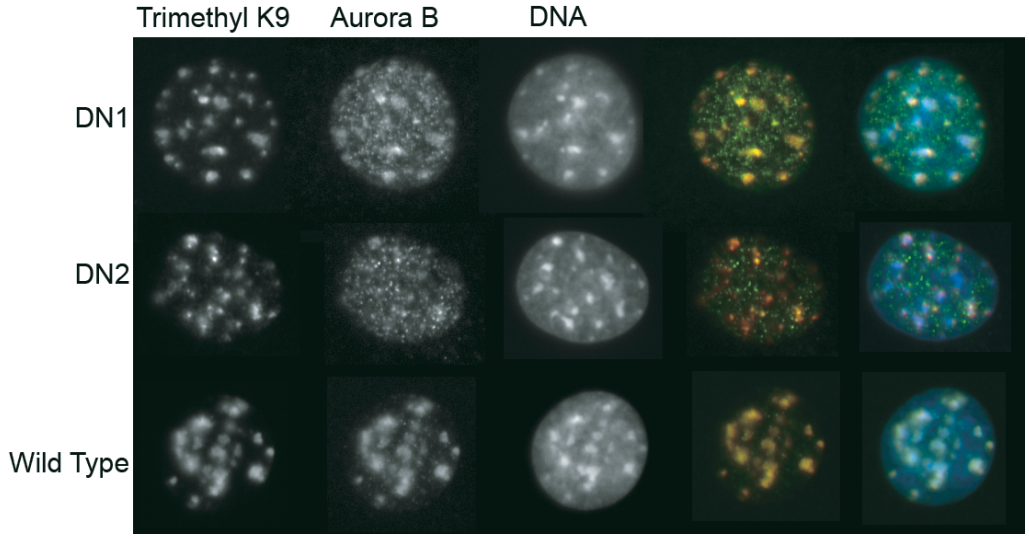


Fig. 4. Aurora B localization in G2 mouse fibroblasts that are wild-type or Suv39h1/Suv39h2 double null. Two Suv39h1/Suv39h2 double-null mouse embryonic fibroblast cell lines and 1 wild-type mouse embryo fibroblast cell line were stained with antitrimethylated K9 and anti-Aurora B. An example of a G2 cell where, in wild-type cells, phosphorylation of serine 10 initiates in pericentromeric heterochromatin and Aurora B is recruited to these sites. The first composite shows the staining of Aurora B (green) and trimethylated lysine 9 (red). The second composite also includes 4′,6-diamidino-2-phenylindole stained chromatin (blue).



The phenotype of these cells includes elongated telomeres, an apparent defect in the release of cohesion at the centromeres, and genomic instability with a tendency to increase chromosome number with increased passage (Gonzalo et al. 2005). This phenotype parallels what has been reported for the Suv39 double null mice (Peters et al. 2001). Importantly, cells lacking all Rb family members, unlike those from Suv39 double-null mice, maintained histone H3 lysine 9 trimethylation in pericentromeric heterochromatin (Gonzalo et al. 2005). Although it has not been directly tested, it is likely that the retention of this modification would allow proper localization of HP1 proteins, even in the absence of trimethyl K20. This implies the existence of a lysine 20 methylation-dependent regulation of cohesin binding or degradation that operates independently of HP1. In this case, the absence of lysine 20 trimethylation contributes to increased stability of cohesins that are associated with pericentromeric heterochromatin. It is important to note, however, that the loss of lysine 9 trimethylation in pericentromeric heterochromatin results in the failure to properly target histone H4 lysine 20 trimethylation (Schotta et al. 2004). Thus, cells deficient in Suv39 lack both lysine 9 trimethylation and lysine 20 trimethylation in pericentromeric heterochromatin.

The dynamics of pericentromeric heterochromatin during mitosis

In early G2, the pericentromeric heterochromatin is phosphorylated by the Aurora B kinase (Adams et al. 2001*b*; Fischle et al. 2005; Zeitlin et al. 2001). This targeting occurs as part of the INCENP–survivin–Aurora B complex (Adams et al. 2001*a*). The resulting phosphorylation of serine 10 correlates with the displacement of HP1α, HP1β, and HP1γ, all reaching a maximum in metaphase, although a small amount of HP1α is retained near the centromeres (Bartova et al. 2005; Fischle et al. 2005; Hirota et al. 2005). A question that then arises is whether HP1 displacement is necessary to enable cohesin dissociation following cleavage by the anaphase promoting complex. Although the displacement of HP1 by serine 10 phosphorylation implies impor-

tance, mutation of this residue to alanine causes segregation defects in *Schizosaccharomyces pombe* that are more consistent with an inability to load condensin onto pericentromeric heterochromatin (Mellone et al. 2003). Moreover, restricting the function of lysine 9 trimethylation to the recruitment of HP1 and, thus indirectly cohesin, is difficult to reconcile with the cell-cycle-dependent changes in histone H3 lysine 9 methylation in pericentromeric heterochromatin (Fig. 3). This post-translational modification increases 4-fold from early G2, when the phosphorylation of serine 10 is initiated, to metaphase, when the phosphorylation of serine 10 is widespread throughout the genome (McManus et al. 2006). Although there is some retention of HP1α at these sites, the binding of HP1 is reduced relative to the levels observed during interphase (Bartova et al. 2005).

A possible explanation for these observations is the behavior of the Aurora B kinase in the absence of lysine 9 trimethylation. Specifically, Aurora B is not properly targeted to pericentromeric heterochromatin during G2 (Fig. 4) and the initiation of serine 10 phosphorylation is spatially and temporally altered relative to wild-type cells. This raises the possibility that some of the mitotic defects observed in the absence of histone lysine 9 trimethylation are a result of a reduction in Aurora B kinase activity in this domain. In the absence of Aurora kinase activity in *Drosophila*, histone H3 is not phosphorylated during mitosis (Adams et al. 2001*b*), and this correlates with an increase in the number of lagging chromosomes observed in anaphase figures (Adams et al. 2001*b*; Giet and Glover 2001). It also correlates with an inability to recruit condensin, which compacts the metaphase chromosome (Giet and Glover 2001). Thus, we must consider the possibility that some of the mitotic defects observed in the absence of histone H3 lysine 9 trimethylation are a result of a reduction in Aurora B kinase activity in this domain.

From molecular biochemistry to three-dimensional structure

The principal determinant of centromere formation is the replication-independent incorporation of CENP-A, a histone H3 homolog, into the sequences that assemble the centromere. Centromeres contain clusters of nucleosomes that contain CENP-A, which are interspersed with clusters of nucleosomes that contain histone H3 (Sullivan and Karpen 2004). Nonetheless, in 3 dimensions, these domains remain segregated into a discrete chromatin structure throughout the cell cycle (Sullivan and Karpen 2004). The properties to specify the formation of such a structure, it would seem, reside in the N-terminal domain of CENP-A. While this domain shows the greatest divergence with the major histone H3 subtypes, it also shows considerable length and sequence variability among species. This remarkable conservation of function, exemplified by the functional replacement of the CENP-A homologue in human cells that were knocked down using RNAi with Cse4 from *Saccharomyces cerevisiae* (Wieland et al. 2004), can be explained by the intrinsic disorder hypothesis of histone tail function recently proposed by Hansen and colleagues (Hansen et al. 2006). Like the C-terminal domain of histone H1 and the N-terminal domains of the core histones, the N-terminus of CENP-A contains a very high proportion of amino acids that favor a disordered state in solution. By coupling the adoption of a specific three-dimensional structure with the binding of an interacting partner, proteins with this property are able to generate considerable structural diversity through a short sequence of amino acids. In this context, the N-terminus of CENP-A may have diverged to accommodate different DNA sequence environments, as proposed by Henikoff and colleagues (Ahmad and Henikoff 2001; Malik et al. 2002), while maintaining the capacity to engage in multiple specific interactions with kinetochore proteins, as well as potential interactions with DNA. One mechanism to specify the segregation of nucleosomes that contain CENP-A from those that contain H3 would be the self-association of CENP-A tails. The diversity of structure and associations enabled by an intrinsically disordered domain could allow all of these interactions to occur despite the small size of the CENP-A tails in many species.

Our understanding of the relationships between centromere structure and function, in contrast with the biochemical regulation of centromere function, is still in its infancy. The centromere is consistently evident as a distinct chromatin structure with a decreased diameter and the site of interaction between chromatids when visualized in situ or in chromosome spreads. The precise structure–function relationship within the centromere and the contribution of histone post-translational modifications to the formation of centromeric and pericentromeric heterochromatin is largely undetermined. Given that biophysical experiments have clearly established that the sensing of tension is critical to the control of mitosis, it might be expected that any significant change in centromeric or pericentromeric heterochromatin structure would compromise the function of the centromere and (or) kinetochore during mitosis. We may be nearing the time when all of the direct biochemical players in this process have been identified. Nonetheless, a list of the parts and the study of small complexes in vitro are a long way from a complete understanding of how the massive three-dimensional structures, which make up the centromere and kinetochore of higher eukaryotes, are assembled from these parts. For this, we will need a much better understanding of the quantitative relationships among post-translational modifications of histones, the recruitment of HP1 and cohesins, the mechanical properties of chromatin during mitosis, and the composition of the kinetochore. This level of understanding will require a very different approach from what has been used to assign molecules in a binary fashion as either required or not.

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