Reinterpreting pericentromeric heterochromatin

Christopher N Topp[1] and R Kelly Dawe[1,2]

In fission yeast, pericentromeric heterochromatin is directly responsible for the sister chromatid cohesion that assures accurate chromosome segregation. In plants, however, heterochromatin and chromosome segregation appear to be largely unrelated: chromosome transmission is impaired by mutations in cohesion but not by mutations that affect heterochromatin formation. We argue that the formation of pericentromeric heterochromatin is primarily a response to constraints on chromosome mechanics that disfavor the transmission of recombination events in pericentromeric regions. This effect allows pericentromeres to expand to enormous sizes by the accumulation of transposons and through large-scale insertions and inversions. Although sister chromatid cohesion is spatially limited to pericentromeric regions at mitosis and meiosis II, the cohesive domains appear to be defined independently of heterochromatin. The available data from plants suggest that sister chromatid cohesion is marked by histone phosphorylation and mediated by Aurora kinases.

Addresses
[1] Department of Plant Biology, University of Georgia, Athens, Georgia 30602, USA
[2] Department of Genetics, University of Georgia, Athens, Georgia 30602, USA

Corresponding author: Dawe, R Kelly (kelly@plantbio.uga.edu)

Current Opinion in Plant Biology 2006, **9**:647–653

This review comes from a themed issue on
Cell biology
Edited by Laurie Smith and Ulrike Mayer

Available online 2nd October 2006

1369-5266/$ – see front matter

DOI 10.1016/j.pbi.2006.09.008

Introduction

The chromatin domains that flank centromeres are known as pericentromeric heterochromatin, pericentric heterochromatin, or simply as pericentromeres. Pericentromeres are the primary sites of sister chromatid cohesion, which is necessary for proper orientation of paired kinetochores during cell division. There is a long history of proposing functional relationships between heterochromatin and cohesion (e.g. [1,2]) but the strongest data come from recent years, *Schizosaccharomyces pombe*. In this fission yeast, an interplay of weak repeat transcription, double-stranded DNA (dsDNA) formation and processing, and short interfering RNA (siRNA)-directed histone modification creates a biochemically defined pericentric heterochromatin domain [3,4]. Virtually any disturbance of the heterochromatic state in *S. pombe* results in severe cell division defects due to loss of cohesion [3,4]. In plants, however, recent data suggest there is very little functional relationship between heterochromatin and cohesion. Here, we review these data and present our perspectives on the origin of heterochromatin and the cell biology of chromosome segregation.

The role of the functional pericentromere in cell division

Accurate chromosome segregation requires a series of timely molecular events. Chief among these are the deposition and removal of cohesin complexes that mediate the association of sister chromatids during mitosis and meiosis. Cohesins consist of four subunits that are thought to form ring structures that link DNA and align replicated chromosomes along their lengths [5,6]. Cohesin facilitates chromosome inheritance in two important ways: first, it ensures that sister kinetochores attach to the correct spindle-poles, and second, it resists the pulling force generated by the spindle until anaphase [6,7•,8].

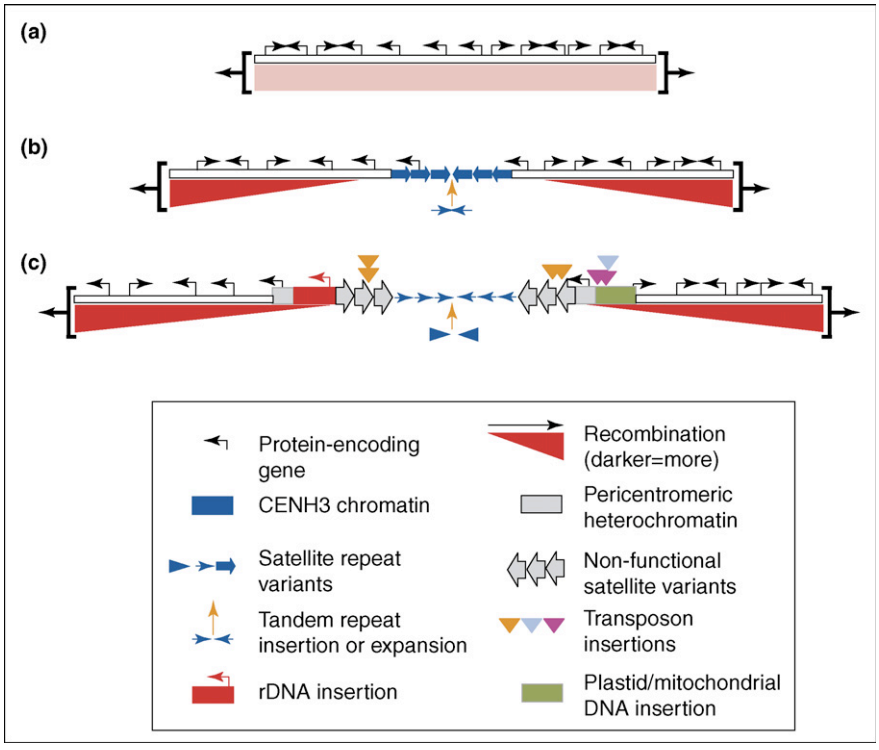
Operationally, pericentromeres are best defined by the retention of cohesin at metaphase. Although cohesin is initially installed throughout the genome in vertebrates, most of the cohesin along chromosome arms is marked by kinases and removed prior to metaphase. Once the proper checkpoints are cleared, pericentric cohesins too are phosphorylated and cleaved by kleisin or separase, releasing each chromosome to the poleward pull of the spindle [9]. These basic processes and core machinery appear to be conserved in plants [7•,9,10•,11–14].

The evolutionary biology of pericentromeres

Unlike their compact and genetically stable yeast counterparts, plant pericentromeres are ill-defined and genetically labile [15]. These traits have given pericentromeres a reputation as genomic 'junkyards': silent repositories of repetitive DNA and other useless DNA elements. To some extent this view is probably correct, but the large reservoirs of DNA within pericentromeres might also contribute to the evolution of new genes and new forms of gene regulation.

The portion of pericentromeres that most resembles 'junk' is that derived directly from centromeres. Centromeres are readily differentiated from pericentromeres by the presence of Centromeric Histone H3 (CENH3), a histone H3 variant that mediates the formation of kinetochores. The primary centromeric DNAs are tandem

Figure 1



A theoretical view of pericentromere evolution. **(a)** Centromeres are known to move to new positions. In animals, such 'neocentromeres' frequently occur in euchromatin (shown here) with a normal distribution of genes. **(b)** Once a centromere is formed, tandem repeats tend to accumulate in the CENH3-marked area. The presence of a new centromere is probably sufficient to recruit cohesin to flanking pericentromeric regions in the absence of heterochromatin. Recombination events near the new centromere are suppressed or poorly transmitted (Figures 2 and 3). **(c)** Low-recombination areas flanking centromeres accumulate selfish DNA and other large insertions. These large tracts of non-coding regions are subject to gene silencing, and the resulting heterochromatin might suppress recombination further.

repeat arrays, which contract and expand rapidly [16]. Rapid centromere turnover causes older repeat arrays to be displaced into the flanking pericentromeres, where they lose their association with CENH3 [17,18]. Over time, these 'discarded' centromere repeats degenerate and become lost in the larger sea of repeats, duplications, and insertions that make up the bulk of plant pericentromeric DNA (Figure 1).

Sequence comparisons among closely related plant species can be used to infer the evolutionary history of pericentromeres. Analyses of four Brassicaceae members revealed that, despite an overall genome contraction in the past 5–15 million years, *Arabidopsis* pericentromeres have doubled in size due to transposable elements, 5S rDNA, and putative gene insertions [19•]. The homology of these regions to *Arabidopsis thaliana* euchromatin suggests that pericentromeres might have a role in seeding new gene creation, as suggested in animals, where segmental duplications are commonplace [19•,20]. Similarly, rice pericentromeres are hotspots for large insertions of chloroplast DNA that are reshuffled and rapidly deleted [21].

Despite the potential for new gene formation, pericentromeres are relatively gene-poor (Figure 1). Pericentromeric heterochromatin is especially prevalent in tomato, in which it encompasses over 75% of the genome [22,23]. A recent cytological analysis of sequenced tomato bacterial artificial chromosomes (BACs) revealed that there are around 8 times fewer genes in pericentromeres than in distal euchromatin [22]. By contrast, *Arabidopsis* pericentromeric heterochromatin represents only a small fraction of the physical genome and has about 50 times fewer genes than average euchromatic regions [22]. These data suggest that the content, size and dynamics of pericentric heterochromatin are species-specific.

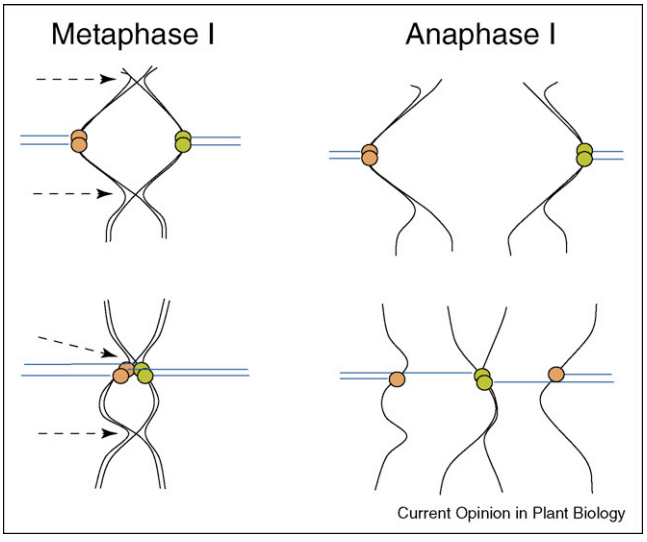
The origin of heterochromatin

What factors contribute to the expansion of pericentromeres and their associated rapid evolutionary change? This question can be recast in terms of recombination suppression because recombination is thought to be a primary force in the removal of unnecessary sequences: when recombination is reduced by any mechanism, repeats are expected to accumulate ([24]; Figure 1). It is possible that cohesin suppresses recombination, but we

know of no data from multicellular eukaryotes that would support such a role. Heterochromatin itself is thought to restrict recombination in some species (e.g. [25,26]), although in rice, a recent analysis of pericentromeres [27•] showed no correlation between recombination and several common histone modifications (H3K4me, H4Ac and H3K9me2; although see also [28]). Even if heterochromatin does restrict recombination, there is a circularity to the argument that heterochromatin suppresses recombination, which in turn allows the accumulation of heterochromatin.

One explanation for the expansion of pericentromeric regions is shown in Figure 2. In yeast, *Drosophila* and humans, recombination close to centromeres often results in chromosome loss [29]. This observation might have its roots in the way that chromosomes align on the metaphase plate at meiosis I to ensure that homologous chromosomes (not sister chromatids) segregate to opposite poles. If recombination occurs very close to the centromere, all four kinetochores become closely opposed and the likelihood that sister, not homologous, chromosomes will align to opposite poles increases. By contrast, recombination in the middle of the chromosome arms allows a wide separation of homologous kinetochores and promotes their separation (Figure 2). Thus, recombination in the middles and ends of chromosomes should ensure the fidelity of equal reduction, whereas recombination within pericentromeres is likely to result in unequal reduction and chromosome loss. The loss of pericentromeric recombinants would be expected to facilitate the accumulation of the repetitive DNA. We note that this model does not pre-suppose that recombination is suppressed in pericentromeres, only that recombination in pericentromeres, when it occurs, is poorly transmitted.

Figure 2



Chromosome mechanics might effectively limit recombination events to the middles and ends of chromosome arms. Reductional division relies on homologous chromosomes being widely separated at metaphase I. If recombination occurs very close to a centromere, all four kinetochores are restrained in close proximity. Because there are no known marks that distinguish sister from homologous kinetochores, the opportunity for error is presumably much higher (as shown here). This is a speculative model. However, data from several species indicate that when recombination occurs within pericentromeres, chromosomes are likely to missegregate.

The heterochromatin-cohesion connection

It is increasingly apparent that the boundaries of eukaryotic centromeres and pericentromeres are defined by epigenetic mechanisms. Ironically, the key to pericentric silencing is a low level of transcription. Pericentric transcripts are processed into siRNAs by RNA interference (RNAi) machinery and fed into a loop that maintains a heterochromatic state, which in *S. pombe* and animals is defined by methylation at histone H3 lysine 9 (H3K9) and the presence of HP-1/Swi6 (Heterochromatin Protein-1/Switching gene product 6). Mutations in components of the RNAi pathway alleviate pericentromere silencing, and result in severe chromosome segregation defects, apparently because of a loss of cohesion [3,4].

Is there a similar relationship between RNAi, heterochromatin and cohesion in multicellular eukaryotes? A careful analysis of the animal literature reveals a relatively loose, indirect relationship between heterochromatin and cohesion. Loss of crucial RNAi proteins increases centromere repeat transcription and disrupts HP1 localization in chicken, mouse and *Drosophila* [30–33], but chromosome segregation defects were reported only in chicken cells [30]. In mouse cells that lack histone methyltransferases, precocious pericentromere separation occurred but no anaphase defects were noted [34]. These results might suggest that heterochromatin facilitates, but is not necessary for, pericentromeric cohesion.

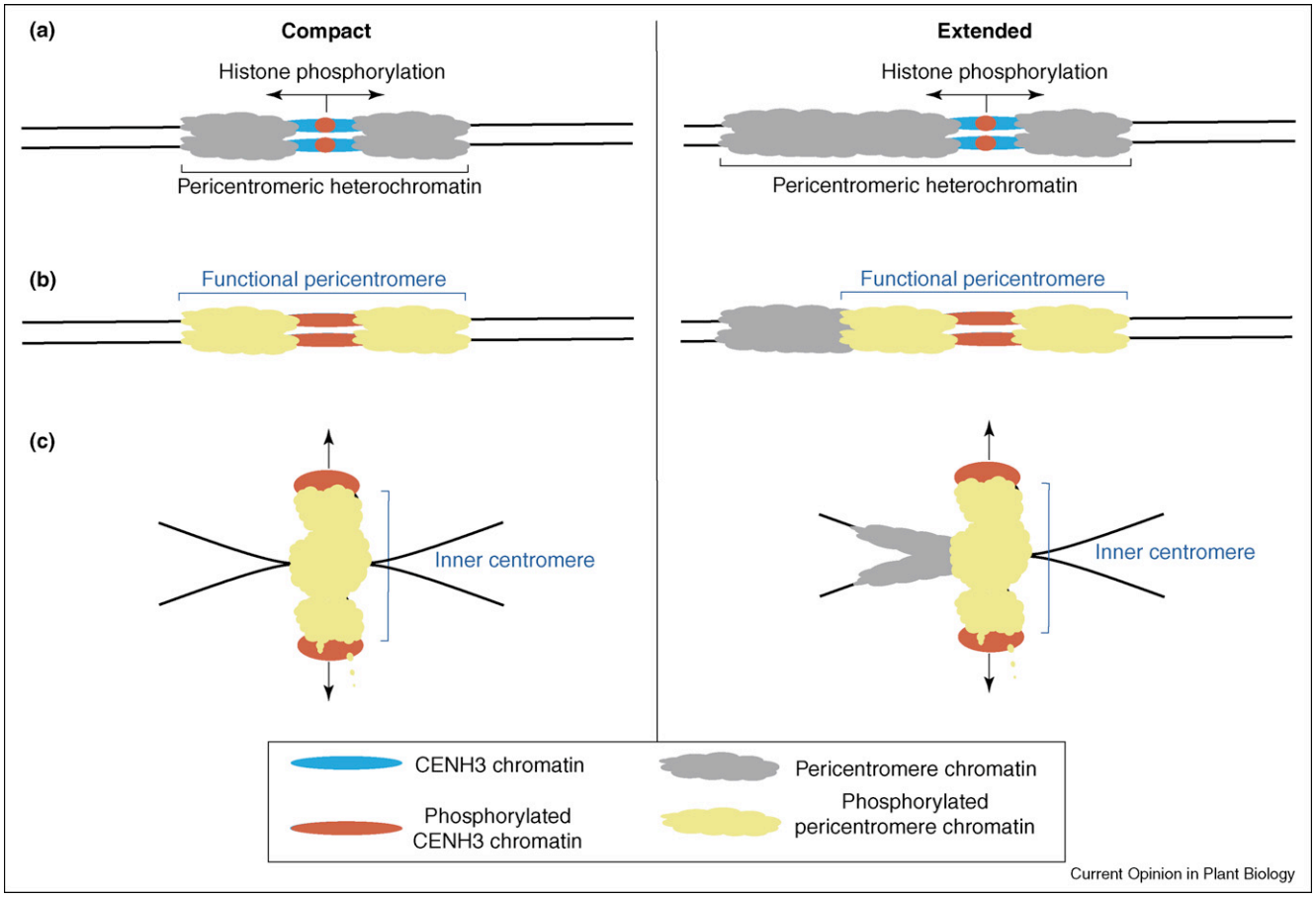
Numerous *Arabidopsis* gene silencing or heterochromatin mutants have also been assayed for defects in chromosome segregation [35••,36••]. In *S. pombe*, RNA polymerase II initiates gene silencing and siRNA production [37], but in plants, a specialized RNA polymerase class (Pol IVa and b) has evolved to facilitate and maintain the heterochromatic state [36••,38,39••]. During interphase, pericentromeric regions including 5S rDNA arrays, nucleolar organizing regions, and other repetitive regions coalesce into deeply 4′,6-diamidino-2-phenylindole, dihydrochloride (DAPI)-staining regions of heterochromatin, known as chromocenters. The *Arabidopsis* homolog of HP1 (LIKE HETEROCHROMATIN PROTEIN 1/TERMINAL FLOWER *2*) appears to be located outside of these chromocenters [40,41]. Mutants of *Arabidopsis* Pol IV cause widespread dispersion of chromocenters and associated reductions in histone

methylation (H3K9me2) and siRNAs [36••,39••]. Despite their profound effect on heterochromatin structure, mutations in Pol IV have no apparent affect on chromosome segregation [36••,39••].

Similar data have come from an analysis of *Arabidopsis* centromere repeat arrays (cen180s) in *Arabidopsis*. Two classes of cen180 repeats were identified: those that are silenced on one strand by an RNAi-heterochromatin process, and those that are silenced on both strands by CpG DNA methylation ([35••]; the repeats analyzed in this study are presumably localized mainly in pericentromeres, not within the CENH3 centromere cores). Interestingly, repeats of the second class appear to be under the control of retroelement long terminal repeats (LTRs). As argued by May *et al.* [35••], the data can be explained if older pericentromere repeats accumulate transposon insertions and become susceptible to transposon-mediated silencing. Similar regulation is likely in other plant species as siRNAs corresponding to the major centromeric repeats have also been reported in *Oryza* species [42] and found in maize (CN Topp, RK Dawe, unpublished). On the basis of these data, we might predict that (as in *S. pombe* [4]) mutations that affect RNAi might also affect chromosome segregation. However, despite substantial loss of H3K9me2 from centromere repeats and transposons in RNAi mutants (e.g. *argonaute 4* [*ago4*] and *kryptonite* [*kyp*]) and DNA methylation mutants (e.g. *defective DNA methylation 1* [*ddm1*]), no abnormal cell division or growth defects are apparent. By contrast, mutations in the genes that encode plant cohesin subunits cause severe chromosome segregation defects [14,35••,43].

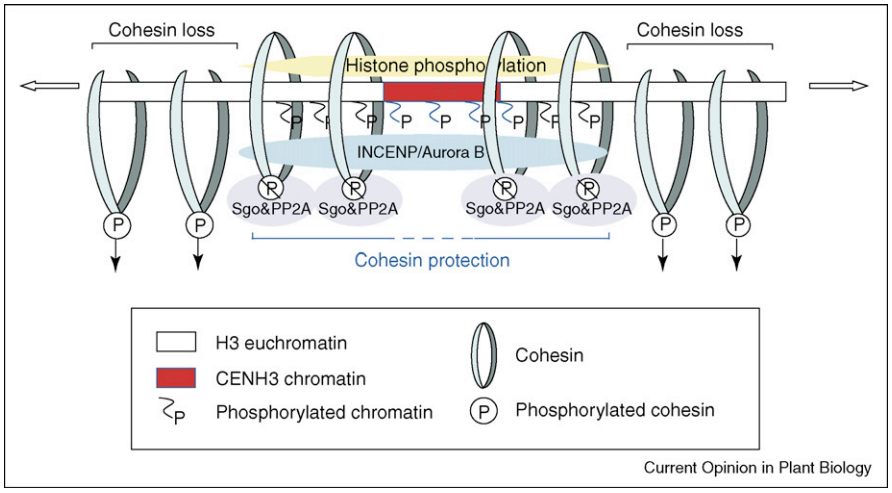
Taken together, the available data suggest that, in multicellular eukaryotes and in plants in particular,

Figure 3



Distinguishing the functional pericentromere from pericentromeric heterochromatin. Pericentromeric heterochromatin is ill-defined and varies among organisms from compact (as in *Arabidopsis*; left) to extended (maize and tomato; right) [22,38]. **(a)** In plant prometaphase, CENH3 is phosphorylated (purple) [49••], **(b)** Phosphorylation spreads to pericentromeric histones on residues H3S10 and H3S28 (green) [43,45,48,49••,50], **(c)** The phosphorylated domain appears to delimit the cohesive regions between sister kinetochores. The compact pericentromeric heterochromatin of *Arabidopsis* (left) might coincide with the cohesive domain. In species such as tomato and maize [28,49••], only a portion of the extended pericentromeric heterochromatin can participate in sister chromatid cohesion.

Figure 4



A model for heterochromatin-independent pericentromere formation. CENH3 is a constitutive feature of all centromeres and is known to be phosphorylated in a cell-cycle-specific manner. The phosphorylation is presumably catalyzed by Aurora kinase [56]. Zhang *et al*. [49••] proposed that once CENH3 is phosphorylated, Aurora kinase subsequently phosphorylates pericentric histones and marks the functional pericentromere ([49••,56]; Figure 3). Aurora kinase could then recruit Shugoshin (Sgo), which has cohesin-protecting properties (summarized by [54]). The interaction between Aurora and Sgo is presumably indirect, as in *Drosophila*, where Aurora B cooperates with INCENP to recruit Sgo. In *S. cerevisiae* and in vertebrates, shugoshin protects cohesin in part by recruiting phosphatase PP2A. The model is speculative and incorporates data from humans, maize, *Drosophila* and yeast.

heterochromatin does not have the direct cohesin-recruiting function that it does in *S. pombe*. How then is cohesin recruited and chromosome segregation regulated?

Histone phosphorylation as an epigenetic mark for cohesin deposition

During cell division in many organisms, pericentromeric chromatin is phosphorylated at conserved histone H3 residues serine10 and 28 (H3S10ph and H3S28ph) by Aurora kinases, which are key regulators of the transition from metaphase to anaphase and the release of chromatids [44–46]. These phosphorylation events are thought to be important for chromosome condensation [47], and in plants are strongly correlated with cohesion [43,48,49••]. Maize plants that carry mutations in the meiotic cohesin subunit Absence of First Division 1 (AFD1) undergo early reductional division and lack H3S10 phosphorylation [11,43]. Furthermore, in *Arabidopsis* and maize, a tight spatial and temporal relationship exists between pericentric H3S10ph, H3S28ph, and sister chromatid cohesion. These two histone modifications define sharply demarcated domains between sister kinetochores at metaphase II ([48,49••]; Figure 3). It has been proposed that the discrete pericentromeric localization of H3S10ph and H3S28ph in plants is initiated during CENH3 phosphorylation [49••].

This idea is supported by the fact that newly originated yeast and mammalian artificial chromosomes recruit cohesin *de novo* (e.g. [50–52]). In human cells, the localization and function of Aurora kinases are closely tied to a protein known as Inner Centromere Protein (INCENP), which is present at active centromeres. It is not yet clear whether INCENP is present in plants. INCENP recruits Aurora B, which is in turn required for the localization of a protein known as shugoshin (at least in *Drosophila* [53]). Shugoshins are a conserved family of proteins that are required for the maintenance of pericentromeric cohesin [9,10•], presumably through the activity of a specific phosphatase (as in yeast, see [54]).

It seems plausible that a similar scenario occurs in plants, and that the process is initiated in some manner by CENH3 and Aurora kinases. The regions identified by H3S10ph and H3S28ph in plants are strikingly similar to the staining pattern of INCENP in human mitotic cells [49••,55]. Further, at least one variant of Aurora kinase localizes specifically to pericentromeric regions in *Arabidopsis* [45]. As suggested by Zhang *et al.* [49••], CENH3 might have a role in recruiting Aurora kinase and facilitating its spread over flanking chromatin. Shugoshin would be expected to follow in the trail of INCENP (or another centromere factor) and Aurora kinase, and to maintain cohesin in centromere-proximal domains (Figure 4). Under this view, cohesin-rich domains are defined epigenetically, but by histone phosphorylation rather than by histone methylation as in *S. pombe* (Figure 3).

Acknowledgements

Work in the corresponding author's laboratory is supported by a grant from the National Science Foundation (0421671).

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