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Focus Review

Focus on the centre: the role of chromatin on the regulation of centromere identity and function

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The centromere is a specialised chromosomal structure that regulates faithful chromosome segregation during cell division, as it dictates the site of assembly of the kinetochore, a critical structure that mediates binding of chromosomes to the spindle, monitors bipolar attachment and pulls chromosomes to the poles during anaphase. Identified more than a century ago as the primary constriction of condensed metaphase chromosomes, the centromere remained elusive to molecular characterisation for many years owed to its unusual enrichment in highly repetitive satellite DNA sequences, except in budding yeast. In the last decade, our understanding of centromere structure, organisation and function has increased tremendously. Nowadays, we know that centromere identity is determined epigenetically by the formation of a unique type of chromatin, which is characterised by the presence of the centromere-specific histone H3 variant CenH3, originally called CENP-A, which replaces canonical histone H3 at centromeres. CenH3-chromatin constitutes the physical and functional foundation for kinetochore assembly. This review explores recent studies addressing the structural and functional characterisation of CenH3-chromatin, its assembly and propagation during mitosis, and its contribution to kinetochore assembly.

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Introduction

The centromere is a highly differentiated chromosomal structure that fulfils multiple functions during cell division, governs kinetochore assembly and ensures equal chromosome segregation to daughter cells during mitosis and meiosis. Apart from its intrinsic biological interest, the analysis of the centromere is also relevant from a biomedical perspective, as alterations in centromere function lead to aneuplody (gain or loss of chromosomes) that most frequently results in lethality. Aneuploidy is also a common event in various diseases, both congenital and acquired, including tumour progression, infertility and birth defects. Centromere biology has been a very intense area of research in the past few years and, though still incomplete, a general picture of the centromere, its structure and regulation, is emerging.

Eukaryotic centromeres come in quite different flavours (reviewed in Sullivan *et al*, 2001; Allshire and Karpen, 2008) (Figure 1). Monocentric eukaryotes contain 'localised' centromeres, in which centromere formation is restricted to a specific chromosomal *locus*. On the other hand, in holocentric organisms such as the nematode *Caenorhabditis elegans*, a 'diffuse' centromere forms along the entire chromosome. In addition, 'localised' centromeres are highly variable in size and sequence, ranging from the simple 'point' centromeres of the budding yeast *Saccharomyces cerevisiae* to the large 'regional' centromeres found in the fission yeast *Schizosaccharomyces pombe*, the pathogenic fungus *Candida albicans*, *Drosophila*, plants and human cells. In the 16 chromosomes of *S. cerevisiae*, centromeric function resides in an essential 125 bp long *CEN* sequence that comprises three conserved functional elements (CDEI, II, III). However, this quite simple sequence-based organisation of *S. cerevisiae* centromeres is not conserved in most monocentric eukaryotes that, instead, contain large 'regional' centromeres. In *S. pombe*, centromeres localise to 40–100 kb long regions consisting of non-homologous 4–5 kb central-core sequences flanked by repeated *imr* and *otr(dg/dh)* elements present in the three *S. pombe* chromosomes. Centromeres of *C. albicans*, though not embedded in long tracts of repetitive DNA, show a similar structural organisation, in which a core sequence is located near short inverted/direct repeats. In *Drosophila*, centromeres are also located in repeated DNA. Actually, the only *Drosophila* centromere characterised at the DNA level corresponds to a 420 kb long region composed of tandem arrays of short satellite DNA repeats interrupted by transposable elements. Similarly, in humans cells, centromeres consist of long α-satellite arrays extending for 0.1–4 Mb. Plant centromeres too are 'regional' containing variable amounts of tandem arrays of satellite repeats and transposable elements.

Lack of conservation of centromeric DNAs suggests that DNA sequence is not the main determinant of centromere identity and function. Actually, centromeric DNAs are neither necessary nor sufficient to support centromere function. On one hand, in stably transmitted dicentric chromosomes, one of the two centromeres is functionally inactivated, indicating that the presence of centromeric DNA does not necessarily lead by itself to the formation of a functional centromere

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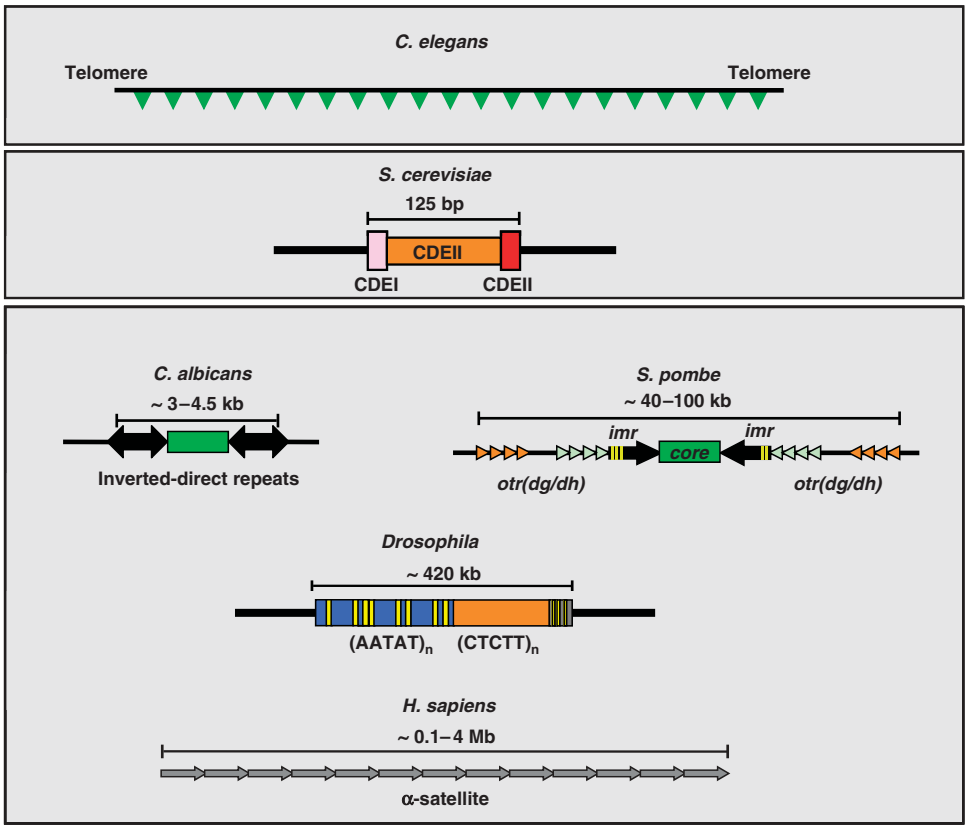

Figure 1 Structural organisation of the different classes of eukaryotic centromeres. In holocentric organisms (*C. elegans*), centromeres form along the entire chromosome. Most eukaryotes, however, contain monocentric chromosomes, in which the centromere forms at a single, generally large, chromosomal region (*C. albicans*, *S. pombe*, *Drosophila*, *H. sapiens*). In *S. cerevisiae*, centromeric function resides in a small 125 bp long conserved DNA sequence. See text for details.

(Earnshaw and Migeon, 1985; Sullivan and Schwartz, 1995; Warburton *et al*, 1997; Agudo *et al*, 2000). Moreover, the formation of neocentromeres at non-centromeric sites shows that, under some circumstances, non-centromeric DNAs can also acquire centromere activity (Williams *et al*, 1998; Choo, 2001; Lo *et al*, 2001; Warburton, 2004). All these observations led to the proposition that, rather than by the DNA sequence itself, centromere identity and function is regulated epigenetically through the formation of a specialised chromatin structure.

As a matter of fact, centromere structure and organisation shows a high degree of conservation at the chromatin level, as centromeric chromatin shares a number of common features. In particular, all eukaryotic centromeres, from *S. cerevisiae* to humans, are characterised by the presence of the centromere-specific histone H3 variant, CenH3 (Earnshaw and Migeon, 1985; Palmer *et al*, 1991; Meluh *et al*, 1998; Buchwitz *et al*, 1999; Henikoff *et al*, 2000; Takahashi *et al*, 2000). In addition, most centromeres map to highly condensed heterochromatic regions. CenH3-containing chromatin and heterochromatin form structurally and functionally distinct domains. For instance, in *S. pombe*, the central-core region is characterised by the presence of the centromere-specific CenH3 variant, $CenH3^{Cnp1}$. On the other hand, *imr/otr*-regions show the characteristic features of heterochromatin, enrichment in H3K9me2 and binding of HP1(Swi6). Boundary elements, encoded by tRNA genes, insulate inner CenH3-chromatin from flanking heterochromatin. More complex 'regional' centromeres also seem to adopt a similar structural organisation (Sullivan and Karpen, 2004). These two distinct domains, which are both required for proper chromosome segregation and inheritance, mediate different functions. CenH3-chromatin is mainly responsible for kinetochore assembly whereas, on the other hand, the surrounding heterochromatin domain seems to have a determinant function in sister-chromatid cohesion. This review focuses on CenH3-chromatin. Aspects related to heterochromatin assembly and function, on the other hand, are out of the scope of this review, but are discussed in another review by Bühler and Gasser (2009), also contained within this focus series.

CenH3-chromatin: distinct composition, structure and organisation

CenH3 is a highly divergent histone H3 variant (Figure 2). The most striking feature of CenH3 resides in the presence of a highly variable N-terminal domain that, ranging in size from 20 to 200 amino acids, shows essentially no sequence homology to the N-tail of histone H3 or across different eukaryotic lineages (Figure 2A). On the other hand, the C-terminal histone-fold domain (HFD) of CenH3, which shows significant homology to histone H3, shares only an average 48% identity across phylogeny (Figure 2B). All these observations indicate that CenH3 evolves very rapidly, which is in contrast to the high evolutionary conservation of

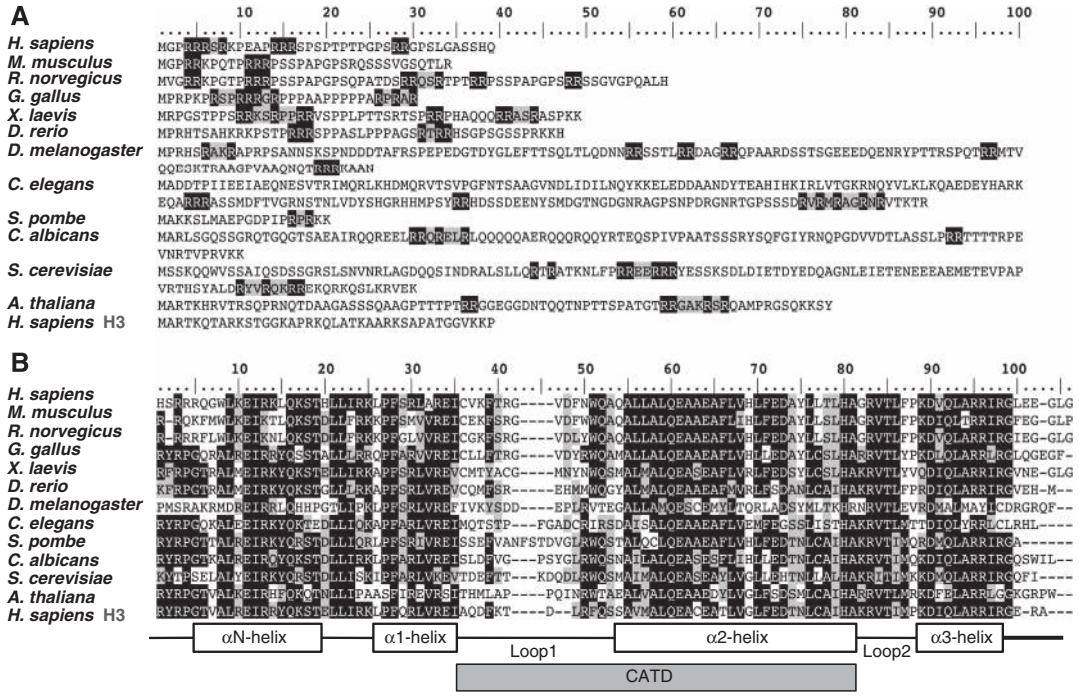

Figure 2 CenH3 is a highly divergent histone H3 variant that evolves very rapidly. Sequence comparison of the N-terminal domain (**A**) and the histone-fold domain (HFD) (**B**) of CenH3 proteins from different species, ranging from *S. cerevisiae* to humans, is shown. The sequence of canonical histone H3 is shown at the bottom for comparison. R-rich motives are indicated in A. Secondary structure of the HFD is indicated in B. The position of the CATD, which mediates centromeric targeting of CenH3 and confers distinct structural properties to CenH3-nucleosomes, is indicated.

canonical histone H3. Fast evolutionary rate of CenH3 is likely reflecting its functional specialisation. Histone H3 interacts with essentially all different classes of genomic DNA sequences and contributes to the regulation of multiple aspects of chromatin structure and function, resulting in strong evolutionary constraints. On the opposite, CenH3 is only required to interact with centromeric DNAs that, being amongst the most rapidly evolving DNA sequences in the genome, seem to drive adaptive evolution of CenH3 in both *Drosophila* and *Arabidopsis* (Malik and Henikoff, 2001; Talbert *et al*, 2002). In mammals, the essential centromere protein CENP-C also shows adaptively evolving domains that overlap with regions of DNA-binding activity though, in this case, there is no evidence for adaptive evolution of $CenH3^{CENP\text{-}A}$ (Talbert *et al*, 2004). Despite their differences, CenH3s from different species share some common structural features. In particular, a consistent feature of CenH3 is the presence of a 2–6 amino acids insertion in the first loop (L1) of the HFD, between helices α1 and α2. A second conserved feature is the presence of arginine (R)-rich motives at the N-tail, which results from a significant enrichment in R-residues in comparison to histone H3.

It is generally assumed that CenH3 incorporates into nucleosomes. The actual composition and structure of CenH3-containing nucleosomes is, however, a matter of debate (Figure 3A). *In vitro* reconstitution experiments showed that human $CenH3^{CENP\text{-}A}$ can replace histone H3 in nucleosomes that, otherwise, show a canonical histone composition and stoichiometry (Yoda *et al*, 2000). In addition, their pattern of DNase I digestion shows a characteristic 10 bp-repeat ladder, similar to what is observed when DNA wraps around a histone octamer. Affinity purification of CenH3-nucleosomes, both from human and fly cells, is also consistent with the formation of 'canonical' (CenH3/H4/H2A/H2B)$_2$ octamers (Blower *et al*, 2002; Foltz *et al*, 2006). Recent results, however, challenged this model. In *S. cerevisiae*, centromeres contain a single $CenH3^{Cse4}$-nucleosome that seems to lack H2A/H2B dimers. Instead, $CenH3^{Cse4}$-nucleosomes contain Scm3, a non-histone protein that is required to recruit $CenH3^{Cse4}$ to centromeres and, *in vitro*, displaces H2A/H2B dimers from preassembled $CenH3^{Cse4}$-containing octamers, suggesting that $CenH3^{Cse4}$-nucleosomes are composed of ($CenH3^{Cse4}$/H4/Scme3)$_2$ hexamers (Camahort *et al*, 2007; Mizuguchi *et al*, 2007; Stoler *et al*, 2007). It is, however, uncertain whether this situation is general, as the presence of Scm3 seems restricted to fungi. Moreover, in *S. pombe*, Scm3 localises at centromeres independently of $CenH3^{Cnp1}$ and dissociates during mitosis (Pidoux *et al*, 2009; Williams *et al*, 2009). On the other hand, on the basis of intranucleosomal cross-linking experiments and atomic-force microscopy measurements, a very provocative model was proposed suggesting that, in *Drosophila*, $CenH3^{CID}$-nucleosomes exist as ($CenH3^{CID}$/H4/H2A/H2B) tetramers, or 'half-nucleosomes', rather than as octamers (Dalal *et al*, 2007a, b). It must be noticed, however, that formation of such heterotypic tetramers is, in general, less favourable than the formation of octamers. It is possible that these alternative models reflect behaviour of CenH3-nucleosomes during dynamic assembly/disassembly processes (see below).

Large sequence deviation from histone H3 strongly suggests that, whether 'canonical' or not, CenH3-containing nucleosomes have differential structural properties. Deuterium exchange experiments indicate that reconstituted

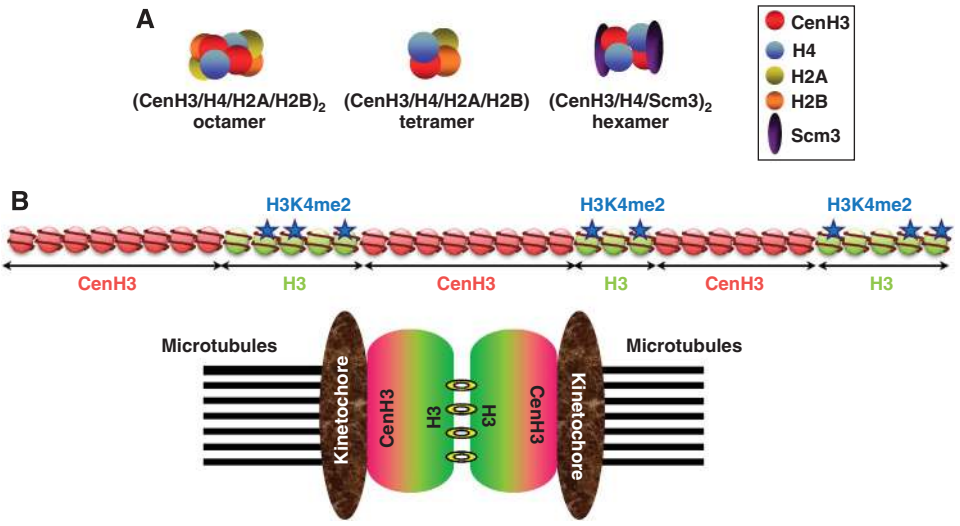

Figure 3 Structural organisation of CenH3-chromatin. (**A**) CenH3-nucleosomes can be composed by $(CenH3/H4/H2A/H2B)_2$ octamers as are canonical nucleosomes. Alternatively, in *Drosophila*, the formation of 'half-nucleosomes', composed by unusual (CenH3/H4/H2A/H2B) tetramers, has been proposed, and, in *S. cerevisiae*, it was reported that H2A/H2B dimers are replaced by Scm3 to form unusual $(CenH3/H4/Scm3)_2$ hexamers. (**B**) In centromeric chromatin, blocks of CenH3-nucleosomes are found interspersed with blocks containing canonical histone H3-nucleosomes. H3-blocks show a peculiar pattern of post-translational histone modifications being hypoacetylated and, at the same time, enriched in H3K4me2. During mitosis, these blocks can direct folding of centromeric chromatin into a specific higher-order structure, in which H3-blocks locate in the interface between sister chromatids and CenH3-blocks face out towards the kinetochore, an arrangement that could facilitate bipolar attachment.

$(CenH3^{CENP\text{-}A}/H4)_2$ tetramers are more compact and rigid than $(H3/H4)_2$ tetramers (Black *et al*, 2004, 2007). These differential structural properties seem to be functionally relevant as they depend on region L1/α2 of $CenH3^{CENP\text{-}A}$, which accumulates many amino acid changes with respect to histone H3 and mediates targeting of CenH3 to centromeres, both in humans and *Drosophila* (Vermaak *et al*, 2002; Black *et al*, 2004). L1/α2 region locates at the predicted interface with histone H4, indicating an important functional contribution of CenH3/H4 interaction. Additional support for this hypothesis comes from genetic analyses in *S. cerevisiae*, as $CenH3^{Cse4}$ was identified as a suppressor of the mitotic defects associated to a specific histone H4 mutation (Smith *et al*, 1996) and, in addition, three out of four identified temperature-sensitive $CenH3^{Cse4}$ mutations map to the predicted $CenH3^{Cse4}$/H4 interface, the fourth mapping to the $CenH3^{Cse4}$/$CenH3^{Cse4}$ interface (Glowczewski *et al*, 2000).

CenH3-chromatin adopts a peculiar organisation, in which blocks of CenH3-nucleosomes are found interspersed with blocks containing canonical histone H3-nucleosomes (Blower *et al*, 2002) (Figure 3B). These blocks, which vary in size from 200 to 500 kb in flies to 500–1500 kb in humans, seem to mediate folding into a specific higher-order structure so that, in mitotic chromosomes, H3-blocks locate in the interface between sister chromatids whereas CenH3-blocks are facing out, towards the kinetochore. This arrangement could facilitate bipolar attachment during mitosis by ensuring that kinetochores form on opposite sites of sister chromatids. Complex regional centromeres of rice, flies, mouse and human adopt this structural organisation. It is, however, uncertain whether the more simple regional centromeres of *S. pombe* and *C. albicans*, which contain a single CenH3-block, or the point centromeres of *S. cerevisiae*, which are formed by a single CenH3-nucleosome, also conform to this model.

Centromeric deposition of CenH3: being in the right place at the right moment

CenH3 exclusively localises to centromeres. Our understanding about how CenH3-nucleosomes are targeted to and assembled at centromeres, though still incomplete, is increasing very rapidly. Differential structural properties of CenH3-nucleosomes seem to have an important contribution to targeting, as centromeric localisation of CenH3 depends on L1/α2 region that, as discussed above, confers distinct structural properties to CenH3-nucleosomes (Figure 2B). Swapping experiments showed that this region, called CATD for CENP-A targeting domain, is necessary and sufficient for centromeric localisation of human $CenH3^{CENP\text{-}A}$ (Black *et al*, 2004), and similar domains have been identified as required for centromeric localisation of CenH3 in both *Drosophila* and *S. cerevisiae* (Keith *et al*, 1999; Vermaak *et al*, 2002; Black *et al*, 2004). How does CATD determine centromeric localisation of CenH3? It is possible that CATD mediates interaction with specific CenH3 recruiting and/or chromatin assembly complexes (see below), which could directly bind to CATD or, alternatively, recognise the distinct structural features of CenH3-nucleosomes. It is also possible that CATD influences stability, and/or specificity, of the interaction with DNA, as loop L1 in histone H3 forms a DNA-binding domain together with loop L2 in histone H4. Swapping experiments performed with $CenH3^{CID}$ from distant *Drosophila* species are consistent with this hypothesis, as centromeric localisation of $CenH3^{CID}$ from *Drosophila bipectinata*, which does not target centromeres in *Drosophila melanogaster*, is restored when L1 of *D. bipectinata* is replaced by that of *D. melanogaster* (Vermaak *et al*, 2002). At this respect, it must be noticed that CenH3 can go to essentially any site on the genome, as shown by transient-expression experiments in different organisms (Van Hooser

et al, 2001; Heun *et al*, 2006; Moreno-Moreno *et al*, 2006). However, when bound to non-centromeric DNA, CenH3-nucleosomes seem to be more unstable (Conde e Silva *et al*, 2007), so that they are evicted and rapidly degraded by proteolysis (Moreno-Moreno *et al*, 2006). On the contrary, CenH3-nucleosomes are tightly bound at centromeres, showing an extremely low turnover (Hemmerich *et al*, 2008). In addition, though neocentromeres can form over different types of DNA sequences, having no similitude to centromeric DNAs, some chromosomal regions seem to be more prone to form neocentromeres in humans (Marshall *et al*, 2008).

Contrary to canonical histones, which are deposited during DNA replication, CenH3 incorporates into chromatin independently of DNA replication, similar to other histone variants (Shelby *et al*, 2000; Ahmad and Henikoff, 2001) (Figure 4). In human cells, $CenH3^{CENP\text{-}A}$ levels peak in G2 (Shelby *et al*, 1997, 2000), and deposition of new $CenH3^{CENP\text{-}A}$ occurs during mitosis, at late telophase, and early G1 (Jansen *et al*, 2007; Hemmerich *et al*, 2008). In syncytial *Drosophila* embryos, in which no G1/G2-phases are observed, $CenH3^{CID}$ deposition also takes place during mitosis, at anaphase (Schuh *et al*, 2007). Yeasts constitute an exception to this rule as, in *S. cerevisiae*, all pre-existing $CenH3^{Cse4}$ is evicted from centromeres and replaced by newly synthesised $CenH3^{Cse4}$ during S-phase (Pearson *et al*, 2004) whereas, in *S. pombe*, in which G1-phase is exceedingly short, $CenH3^{Cnp1}$ deposition takes place in two phases, during S and in late G2 (Takayama *et al*, 2008). In *Arabidopsis*, it was reported that loading of CenH3 also occurs mainly in G2 (Lermontova *et al*, 2006). As a consequence of its loading outside of S-phase, CenH3 concentration at centromeres is diluted during DNA replication, generating 'gaps' that could remain nucleosome-free, ready for CenH3 deposition during mitosis, or be filled by replicative H3-nucleosomes that, later, would be replaced by CenH3-nucleosomes (Figure 4). It might also be possible that, during DNA replication, CenH3-nucleosomes are disassembled into heterotypic tetramers or 'half-nucleosomes'.

Loading of CenH3 late in mitosis raises some intriguing questions. First, it means that kinetochore assembly actually takes place before centromeres are fully replenished with CenH3-nucleosomes. Second, it also means that loading occurs in close coincidence to chromosome segregation, suggesting that signalling events occurring during segregation might actually trigger CenH3 deposition. And third, it raises the question of how CenH3-assembly could take place during mitosis, when chromatin is believed to be more inaccessible and, in general, refractory to transactions. At this respect, work carried out in *S. pombe* identified a number of factors and complexes that are required for centromeric localisation of $CenH3^{Cnp1}$ (Table I). Amongst those, Mis16/Mis18 complex is required to maintain histone acetylation status at the central-centromere region, indicating that it has a central function in modifying centromeric chromatin (Hayashi *et al*, 2004) (Figure 4). Mis16 is the *S. pombe* homologue of RbAp46/48, a general histone H3/H4 chaperone that forms part of several chromatin assembly, remodelling and modifying complexes. On the other hand, Mis18 is widely conserved in eukaryotes. In humans, hMis18 also cooperates with RbAp46/48 and localises to centromeres only at late telophase/early G1, when newly synthesised CenH3 is deposited (Fujita *et al*, 2007). Similarly, in *S. pombe*, Mis16/Mis18 complex dissociates from centromeres from early mitosis until anaphase, when it again localises to

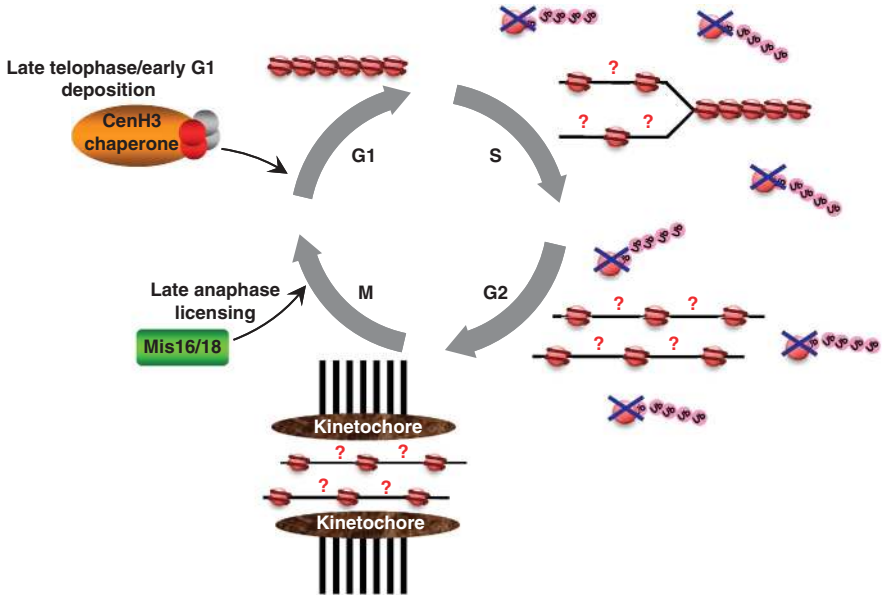

Figure 4 Assembly and dynamic behaviour of CenH3-chromatin during cell cycle. Like other histone variants, CenH3 incorporates into chromatin independently of DNA replication. Deposition of newly synthesised CenH3 takes place during mitosis, at late telophase, or early G1. Specific CenH3 chaperones localise to the centromere coincidentally with deposition of new CenH3 and mediate assembly of CenH3-nucleosomes. During assembly, CenH3 might become resistant to proteolysis that, otherwise, degrades CenH3 and prevents deposition at non-centromeric sites. Before deposition, at late anaphase, specific complexes (Mis16/Mis18) seem to modify centromeric chromatin to allow assembly of new CenH3-nucleosomes. During DNA replication at S-phase, CenH3 concentration at centromeres is diluted and kinetochore assembly takes place before replenishment with new CenH3-nucleosomes. It is unclear whether 'gaps' generated during DNA replication remain nucleosome-free or are filled by replicative H3-nucleosomes. It might also be possible that CenH3-nucleosomes are disassembled into 'half-nucleosomes' to compensate for this deficit.

Table I Factors required for centromeric localisation of CenH3

Type	Factor	Organism	References	Proposed role(s)	Observations
Chaperone	HJURP	*H. sapiens*	Dunleavy *et al* (2009); Foltz *et al* (2009)	CenH3^{CENP-A} deposition	
	Npm1	*H. sapiens*	Dunleavy *et al* (2009); Foltz *et al* (2009)	CenH3^{CENP-A} deposition	
	Sim3	*S. pombe*	Dunleavy *et al* (2007)	CenH3^{Cnp1} deposition	
	CAF-1	*S. cerevisiae*	Sharp *et al* (2002)	CenH3^{Cse4p} deposition	
	RbAp46/48	*H. sapiens*	Hayashi *et al* (2004); Dunleavy *et al* (2009)	Associated to CenH3^{CENP-A} nucleosomes	Mis16–Mis18 complex in *S. pombe* and the RbAp46/48–Mis18 (hMis18α/β–M18BP1) complex in humans regulate histone acetylation at centromeric chromatin
	p55	*Drosophila*	Furuyama *et al* (2006)	Assembly of CenH3^{CID} nucleosomes *in vitro*	
	Mis16	*S. pombe*	Hayashi *et al* (2004)	Licensing centromeric chromatin for deposition	
Chromatin modification/ remodelling	hMis18α/β	*H. sapiens*	Fujita *et al* (2007)	Licensing centromeric chromatin for deposition	
	Mis18	*S. pombe*	Hayashi *et al* (2004)		
	M18BP1	*H. sapiens*	Fujita *et al* (2007)	Licensing centromeric chromatin for deposition	
	KNL2	*C. elegans*	Maddox *et al*, 2007)		
	RSF	*H. sapiens*	Perpelescu *et al* (2009)	Assembly and maintenance of CenH3^{CENP-A}-chromatin	
	Hrp1	*S. Pombe*	Walfridsson *et al* (2005)	Remodelling of centromeric chromatin	Homologous to Chd1 that recognises H3K4me2,3
	Ams2	*S. Pombe*	Chen *et al* (2003)	Transcription of centromeric chromatin	GATA-like transcription factor
Chromatin structural component	Scm3	*S. Pombe*	Pidoux *et al* (2009); Williams *et al* (2009)	Reception of CenH3-nucleosomes	
	Scm3	*S. cerevisiae*	Camahort *et al* (2007); Mizuguchi *et al* (2007); Stoler *et al* (2007)	Structural component of the CenH3^{Cse4}-nucleosome that replaces H2A/H2B dimers	
Kinetochore/ centromere components	Cal1	*Drosophila*	Goshima *et al* (2007); Erhardt *et al* (2008)	CenH3^{CID} assembly	CenH3^{CID}, Cal1 and CENP-C are mutually dependent for localisation
	CENP-C	*Drosophila*	Goshima *et al* (2007); Erhardt *et al* (2008)	Component of NAC. Putative DNA binding protein	Not required in chicken cells (Okada *et al*, 2006)
	CENP-H	*H. sapiens*	Okada *et al* (2006)	Component of NAC. Required for CenH3^{CENP-A} deposition	CENP-H/I and CenH3^{CENP-A} are mutually dependent for localisation
	Sim4	*S. pombe*	Pidoux *et al* (2003)	Component of Mis6/Mis15/Mis17/Sim4	
	CENP-I	*Humans*	Okada *et al* (2006)	Component of NAC. Required for CenH3^{CENP-A} deposition	The *S. cerevisiae* homolog of CENP-I, Ctf13, is not required for CenH3^{Cse4} localisation (Measday *et al*, 2002)
	Mis6	*S. pombe*	Takahashi *et al* (2000)	Component of Mis6/Mis15/Mis17/Sim4	
	CENP-K, -L, -M	*H. sapiens*	Okada *et al* (2006)	Components of NAC. Required for CenH3^{CENP-A} deposition	CENP-K, -L, -M and CenH3^{CENP-A} are mutually dependent for localisation

centromeres. Mis16/Mis18 complex, however, does not seem to directly interact with CenH3. These observations suggest that, rather than directly recruiting CenH3 to centromeres, Mis16/Mis18 complex is involved in modifying centromeric chromatin to allow deposition of new CenH3. Consistent with this hypothesis, treatment with the HDAC-inhibitor TSA restores centromeric localisation of $CenH3^{CENP\text{-}A}$ in the absence of hMis18 (Fujita *et al*, 2007). Additional factors affecting CenH3 localisation might also be involved in licensing centromeric chromatin for deposition of new CenH3.

Recent studies identified chromatin-assembly complexes that mediate incorporation of prenucleosomal CenH3 to centromeres in human cells (Dunleavy *et al*, 2009; Foltz *et al*, 2009) (Figure 4). These complexes contain a unique $CenH3^{CENP\text{-}A}$ histone chaperone, HJURP (Holliday junction-recognising protein), which directly interacts with $CenH3^{CENP\text{-}A}$ and localises to centromeres coincidentally with deposition of newly synthesised $CenH3^{CENP\text{-}A}$. Interaction with HJURP is mediated through the CATD (Foltz *et al*, 2009) and, most interesting, this interaction seems to prevent proteolytic degradation of $CenH3^{CENP\text{-}A}$ as removal of HJURP results, after a few cell divisions, in a strong reduction in $CenH3^{CENP\text{-}A}$ levels (Dunleavy *et al*, 2009; Foltz *et al*, 2009). It must be noted that, both in *S. cerevisiae* and *Drosophila*, proteolytic degradation tightly regulates CenH3 expression to ensure its centromere-only deposition (Collins *et al*, 2004; Moreno-Moreno *et al*, 2006). It is possible that CenH3 proteolysis is also cell-cycle regulated, so that CenH3 becomes resistant to degradation only when it gets incorporated into specific chromatin-assembly complexes. Prenucleosomal CenH3 can also interact with the general histone chaperone RbAp46/48, which is part of CAF-1 and interacts with histone H4 (Furuyama *et al*, 2006; Dunleavy *et al*, 2009), and Npm1 (Nucleophosmin 1), which acts as a chaperone for both H3:H4 and H2A:H2B (Dunleavy *et al*, 2009; Foltz *et al*, 2009). Interestingly, in *Drosophila*, Npm1 acts as an ATP-dependent chromatin remodelling complex (Ito *et al*, 1996), suggesting a link to nucleosome remodelling/exchange. Recently, the contribution of the chromatin remodelling complex RSF to assembly of $CenH3^{CENP\text{-}A}$-chromatin in human cells was reported (Perpelescu *et al*, 2009). In addition, in *S. pombe*, $CenH3^{Cnp1}$ was found to physically interact with Sim3, a NASP-related histone chaperone that was proposed to escort $CenH3^{Cnp1}$ to centromeres (Dunleavy *et al*, 2007). At present, little is known about how CenH3-assembly complexes are recruited to centromeres. They could directly recognise pre-existing CenH3-nucleosomes, specific histone modifications or, alternatively, be recruited through factors that would act as receptor complexes for incoming CenH3. In *S. pombe*, Scm3 was proposed to play such a role, receiving at the centromere incoming $CenH3^{Cnp1}$ from the Sim3 escort chaperone. Scm3 interacts with $CenH3^{Cnp1}$ and is required for its centromeric localisation but not *vice versa* (Pidoux *et al*, 2009; Williams *et al*, 2009). Interestingly, Mis16/Mis18 complex seems to directly recruit Scm3 to centromeres (Pidoux *et al*, 2009; Williams *et al*, 2009), suggesting that, in addition to regulate histone acetylation at centromeric chromatin, Mis16/Mis18 complex might also be involved in recruitment of receptor complexes.

Additional factors are known to be required for centromeric localisation of CenH3, although their precise role in assembly of centromeric chromatin is not fully understood (Table I). These include, some inner kinetochore components (i.e. CENP-C, -H/Sim4, -I/Mis6, -K, -L and -M) that, in turn, are dependent on CenH3 for centromere localisation (Takahashi *et al*, 2000; Pidoux *et al*, 2003; Okada *et al*, 2006; Goshima *et al*, 2007; Erhardt *et al*, 2008). In *Drosophila*, CAL1 also shows reciprocal dependency with $CenH3^{CID}$ and, in addition, with CENP-C (Goshima *et al*, 2007; Erhardt *et al*, 2008). Mutual dependency for centromere localisation between CenH3 and inner kinetochore components indicate the existence of cross-talk mechanisms acting at the initial stages of assembly.

Factors and mechanisms discussed above provide a general picture of how centromere identity is propagated during mitosis. Centromeres, however, are also formed *de novo* in the absence of a pre-existing centromere. The formation of neocentromeres, as well as changes in centromere localisation that occur during evolution, are examples of *de novo* centromere formation (Choo, 2001; Warburton, 2004). *De novo* establishment of centromeres has also been observed in fungi, plants and mammals, when appropriate DNAs carrying centromeric DNA sequences are transfected into cells (Clarke and Carbon, 1980; Hahnenberger *et al*, 1989; Harrington *et al*, 1997; Carlson *et al*, 2007). Recent studies addressed the determination of the requirements for *de novo* centromere formation in *S. pombe*, in which centromere formation on naked DNA templates occurs efficiently only in the presence of heterochromatin (Folco *et al*, 2008). Notice that, in most organisms, centromeres are embedded into large heterochromatic regions. Heterochromatin, however, is not required for centromere maintenance, as mutations disrupting heterochromatin do not impair mitotic propagation of pre-existing centromeres. On the other hand, in mammals, the CENP-B protein also seems to contribute to *de novo* centromere formation (Okada *et al*, 2007). Again, CENP-B is dispensable for centromere maintenance.

CenH3-chromatin and kinetochore assembly: a 'complex' affair

CenH3 is essential for centromere function. In all eukaryotes analysed to date, deletion of CenH3 is lethal, both at the cell and organism level (Stoler *et al*, 1995; Howman *et al*, 2000; Blower and Karpen, 2001; Oegema *et al*, 2001; Régnier *et al*, 2005). Cells deficient in CenH3 fail to localise kinetochore proteins and show strong chromosome segregation defects, indicating that CenH3 is necessary for kinetochore formation. Is it also sufficient? Over-expression experiments performed both in flies and human cells showed that CenH3 misincorporates throughout chromatin (Van Hooser *et al*, 2001; Heun *et al*, 2006; Moreno-Moreno *et al*, 2006) and, although most of it is rapidly removed by proteolytic degradation (Moreno-Moreno *et al*, 2006), a small fraction remains stably associated to a few non-centromeric sites resulting in recruitment of kinetochore proteins at these ectopic sites and, concomitantly, cells showed delayed mitosis, chromosome segregation defects, aneuploidy and growth defects. However, in mammals, only a subset of kinetochore proteins are detected at these ectopic sites, with some essential kinetochore proteins being excluded (Van Hooser *et al*, 2001). Moreover, in flies, not all ectopic sites seem to be equally efficient in recruiting kinetochore proteins and, moreover, spindle attachments could be detected only in a small subset (Heun

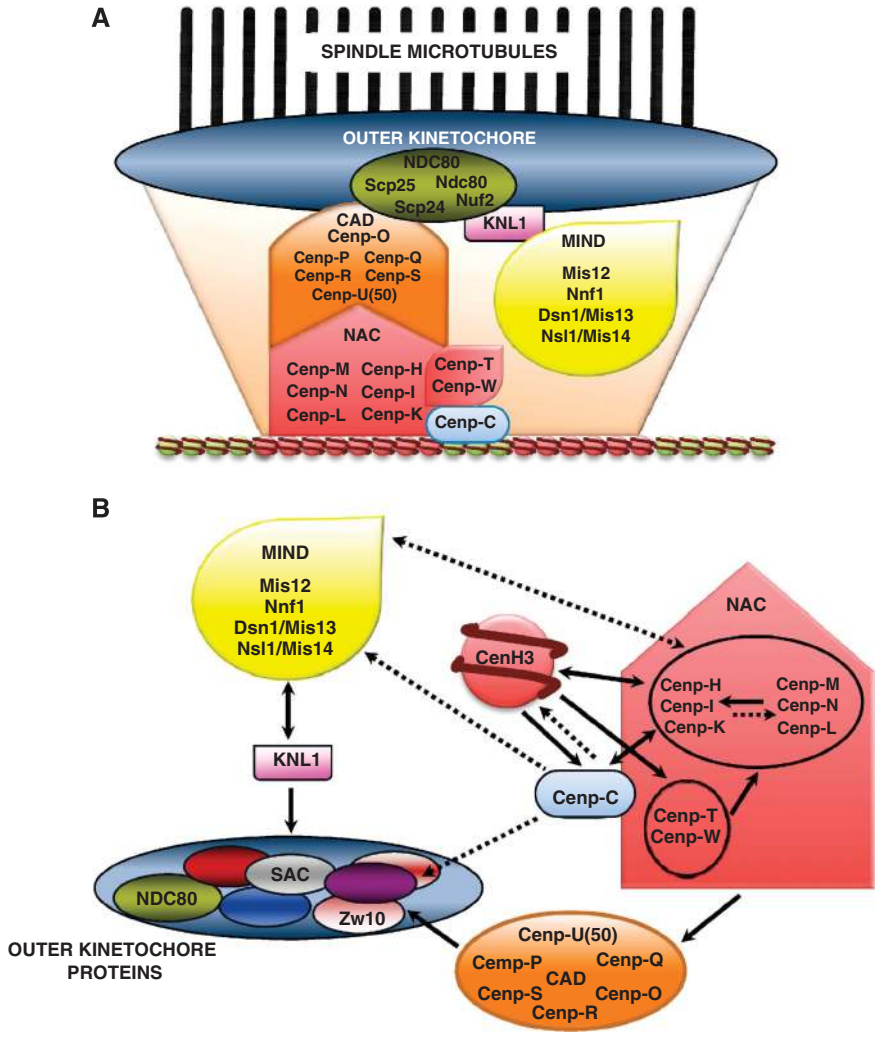

Figure 5 Kinetochores are large macromolecular entities. (**A**) Various protein complexes/networks are known to act at different stages of kinetochore assembly. These include the KNM network (KNL1, NDC80 and MIND), which is involved in microtubule binding, and the NAC/CAD network that directly associates to centromeric chromatin. (**B**) CenH3 is essential for kinetochore assembly. CenH3 is at the bottom of a complex network of interactions that, ultimately, leads to assembly of a fully functional kinetochore. Dependencies for centromeric/kinetochore localisation are indicated by solid arrows. Possible interactions, observed only in some species or not fully confirmed, are indicated by dotted arrows.

et al, 2006). It is likely that not all ectopic sites contain enough CenH3, or in the appropriate organisation, to support formation of a fully functional kinetochore. Alternatively, it is also possible that, independent of CenH3, additional factors have an essential contribution to kinetochore formation. At this respect, it was proposed the existence of a second pathway that, depending on the conserved Mis12 protein, contributes to kinetochore assembly (Goshima *et al*, 1999, 2003). In *S. pombe*, Mis12 is required for equal chromosome segregation, localises to centromeres throughout the cell cycle and, moreover, its centromeric localisation is independent of $CenH3^{Cnp1}$ and *vice versa* (Takahashi *et al*, 2000). A similar situation is observed in human cells, in which depletion of hMis12 results in loss of some kinetochore proteins without affecting centromeric localisation of $CenH3^{CENP-A}$ though, in this case, depletion of $CenH3^{CENP-A}$ seems to partially affect centromeric localisation of hMis12 (Liu *et al*, 2006). As a matter of fact, both in *C. elegans* and *Drosophila*, Mis12 localisation depends on CenH3 (Cheeseman *et al*, 2004; Goshima *et al*, 2007; Przewloka *et al*, 2007).

Kinetochore assembly at the centromere involves complex pathways of hierarchical, sometimes reciprocal, interactions (Figure 5). CenH3 is at the bottom of such network of interactions. At present, we are only beginning to understand its actual contribution to kinetochore assembly. Kinetochores are large macromolecular entities that, depending on the organisms, are composed by dozens to more than a hundred different protein components (for a comprehensive overview, see the accompanying focus review by Santaguida and Musacchio, 2009). Kinetochore architecture is well understood only in *S. cerevisae*, in which binding of a single microtubule requires co-operation of at least six different protein complexes (Mif2, COMA, Spc150, MIND, NDC80 and Dam-DASH), resulting in more than 500 protein molecules participating in formation of the relatively simple *S. cerevisiae* kinetochore, as deduced from quantitative fluorescence microscopy analyses (Joglekar *et al*, 2006). In the rest of eukaryotes, kinetochore composition remains poorly understood. Morphologically, electron microscopic studies show the kinetochore as a trilaminar structure consisting of

an inner-plate, which is in direct contact with centromeric chromatin, and the central- and outer-plates that mediate spindle attachment. Biochemical studies have identified a number of protein complexes/networks that act at different stages of assembly, some being constitutively associated to the centromere throughout the cell cycle, whereas others localise to the kinetochore only transiently during mitosis (Figure 5). Identified complexes include the KNM network (KNL1, NDC80 and MIND), which is involved in microtubule binding, and, in particular, the NAC/CAD network that directly associates to centromeric chromatin (Foltz *et al*, 2006; Okada *et al*, 2006). In addition, a third protein network that regulates chromosome movement, Dam1/DASH, has been identified both in *S. cerevisiae* and *S. pombe*. Components of the NAC/CAD network are good candidates to be directly recruited by CenH3-chromatin. As a matter of fact, in human cells, NAC (nucleosome associated complex) was isolated on the basis of its co-purification with CenH3$^{CENP\text{-}A}$-nucleosomes (Foltz *et al*, 2006). In the same study, CAD (CenH3$^{CENP\text{-}A}$ distal complex) was purified using NAC components as baits. CAD components do not seem to directly associate to CenH3$^{CENP\text{-}A}$-nucleosomes and, moreover, centromeric localisation of some CAD components seems to depend on NAC. NAC/CAD are essential for stabilising microtubule attachments but not for recruitment of some SAC components (Foltz *et al*, 2006). Hierarchical NAC/CAD interactions are, however, complex as NAC components are also required for centromeric localisation of CenH3$^{CENP\text{-}A}$ (Okada *et al*, 2006) and NAC/CAD interactions might be cell-cycle regulated (Kwon *et al*, 2007). In addition, some NAC components (CENP-T/W) might directly bind DNA, as they contain HFDs that mediate DNA-binding *in vitro* (Hori *et al*, 2008). In chicken cells, CENP-T/W seem to preferentially associate to centromeric regions containing canonical histone H3, though their centromeric localisation depends on CenH3$^{CENP\text{-}A}$. Most interestingly, CENP-T/W do not seem to directly influence centromeric localisation of CenH3$^{CENP\text{-}A}$. It must be noticed that CENP-C is also a putative DNA-binding protein (Yang *et al*, 1996).

The molecular basis of the association of NAC/CAD with CenH3-nucleosomes is, however, not known. Which are the components that directly bind CenH3-nucleosomes? What features of CenH3-nucleosomes they recognise? As mentioned above, some NAC components might bind DNA whereas others could recognise CenH3-nucleosomes on the basis on their distinct structural features or directly bind CenH3. Support for this hypothesis comes from studies performed in *S. cerevisiae*, in which deletion of the N-domain of CenH3^{Cse4} causes lethality (Keith *et al*, 1999; Chen *et al*, 2000), which is in contrast to deletion of the N-domain of canonical histone H3 that is viable. Mutation analyses identified within the N-domain of CenH3^{Cse4} a 33 amino acids motif (END) that, being essential for viability, seems to interact with COMA, a kinetochore complex that is functionally related to NAC/CAD and mediates protein–protein interactions with other kinetochore/centromere proteins, including the essential CBF3 complex. Interestingly, END corresponds to an R-rich motif, whose presence at the N-domain is conserved in CenH3s from distant species (Figure 2). It is possible that, also in other organisms, N-domain is involved in interactions with NAC/CAD and/or other kinetochore proteins.

Concluding remarks

In the last few years, we have learned much about the components and mechanisms that determine centromere identity and function. There are, however, several aspects that remain poorly understood. A major challenge for the future is to describe molecularly the pathway(s) leading from centromeric chromatin to assembly of a fully functional kinetochore. To a large extent, the kinetochore is a puzzle that, composed by hundreds of pieces, needs to be pieced together by sorting out individual protein–protein interactions. For this purpose, extensive information needs to be obtained about kinetochore composition and organisation in various organisms by combining both molecular and imaging technologies.

To what extent the structural organisation of CenH3-chromatin changes during cell cycle is another question that needs to be addressed. As mentioned above, centromeric chromatin is actually deficient on CenH3 during a significant part of the cell cycle, from replication of centromeric DNA to deposition of new CenH3. Is this deficit compensated through the deposition of H3-nucleosomes, the formation of 'half-nucleosomes' or, simply, by spacing CenH3-nucleosomes? At this respect, it must be noticed that, in contrast to the regular nucleosomal ladder observed in bulk chromatin, CenH3-chromatin shows a smeared pattern of micrococcal nuclease digestion both in *S. pombe* and *C. albicans*, suggesting a more irregular nucleosomal spacing (Polizzi and Clarke, 1991; Takahashi *et al*, 1992; Baum *et al*, 2006).

It is anticipated that histone modifications would have important functions in regulating centromere biology, as histones are extensively modified post-translationally and covalent histone modifications have an essential contribution to the regulation of chromatin functions, they correlate with different functional states and are involved in chromatin assembly/disassembly processes. Little is known, however, about the actual pattern of post-translational modifications of CenH3-chromatin. Is CenH3 subjected to post-translational modifications? Are they regulated during cell cycle? How? Are they involved in regulating CenH3 deposition, kinetochore assembly or other aspects of centromere/kinetochore function? At this respect, it was reported that human CenH3$^{CENP\text{-}A}$ is phosphorylated at residue S7 in a manner dependent on both Aurora-A and -B (Zeitlin *et al*, 2001; Kunitoku *et al*, 2003). Phosphorylation of human CenH3$^{CENP\text{-}A}$S7 is required for normal progression through mitosis and, unexpectedly, cytokinesis. Expression of non-phosphorylatable mutants disrupts localisation of the chromosomal passenger complex, leads to chromosome misalignment during mitosis and delays cell separation during cytokinesis. Although this residue is not conserved, most CenH3s contain S-residues at the N-domain that, being in a similar sequence context, could be susceptible to phosphorylation by Aurora-A/B.

Investigation of the precise relationship between centromeric chromatin and transcription, and non-coding RNAs, is likely to become an area of intense research in the future. Though centromeres correspond to essentially inactive regions, recent results indicate that centromeric DNAs might actually be transcribed, as a human neocentromere was shown to contain active genes (Saffery *et al*, 2003) and non-coding RNAs homologous to centromeric DNAs have been detected in mammals and plants (May *et al*, 2005;

Bouzinba-Segard *et al*, 2006). Moreover, FACT (facilitates chromatin transcription) complex, which is required for efficient transcription through chromatin, is found associated to human CenH3^{CENP-A}-nucleosomes (Foltz *et al*, 2006), and centromeric H3-blocks, which are found interspersed with CenH3 domains, show a distinct pattern of histone modifications, being enriched in H3K4me2 (Sullivan and Karpen, 2004), a modification normally associated with transcriptionally active chromatin domains. As a matter of fact, several observations indicate that transcription of centromeric chromatin might have a function in assembly of CenH3-chromatin. In *S. pombe*, centromeric deposition of CenH3^{Cnp1} is facilitated by Ams2, a GATA-like transcription factor (Chen *et al*, 2003), and Hrp1 (Walfridsson *et al*, 2005), which corresponds to the fission yeast homologue of Chd1, a chromatin remodelling factor that binds H3K4me2,3. It is also possible that non-coding RNAs themselves, centromeric or not, might physically associate with centromeric chromatin and regulate its structural and functional properties. In fact, early ultrastructural studies suggested the presence of RNA at the kinetochore (Rieder, 1979) and centromere-encoded RNAs have been shown to be integral components of the maize centromere/kinetochore complex (Topp *et al*, 2004).

Finally, it is striking that, despite its highly conserved cellular function, centromeres evolve so rapidly. Not only centromere components are diverse, but also kinetochore proteins seem to evolve adaptively as, most frequently, identifiable orthologs show no significant sequence similarity except for short regions. This high degree of structural diversity likely reflects a contribution to speciation. Epigenetics might facilitate fast evolution of centromeres, as epigenetic regulation permits to bypass strict sequence constraints. The formation of neocentromeres supports this view. The ability to form neocentromeres is conserved through evolution, from fungi to humans, and contributes to karyotype evolution and speciation both in vertebrates and plants. As a matter of fact, recent results indicate that neocentromere formation might be more frequent than previously thought as, in *C. albicans*, neocentromeres can form over multiple chromosomal locations at an extremely high frequency (Ketel *et al*, 2009). Also in *S. pombe*, neocentromeres form very efficiently (Ishii *et al*, 2008). Clearly, it seems as evolutionarily advantageous to 'loosely' regulate centromere identity through epigenetics.

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References

Agudo M, Abad JP, Molina I, Losada A, Ripoll P, Villasante A (2000) A dicentric chromosome of *Drosophila melanogaster* showing alternate centromere inactivation. *Chromosoma* **109:** 190–196

Ahmad K, Henikoff S (2001) Centromeres are specialized replication domains in heterochromatin. *J Cell Biol* **153:** 101–110

Allshire RC, Karpen GH (2008) Epigenetic regulation of centromeric chromatin: old dogs, new tricks? *Nature Rev Genet* **9:** 923–937

Baum M, Sanyal K, Mishra PK, Thaler N, Carbon J (2006) Formation of functional centromeric chromatin is specified epigenetically in *Candida albicans*. *Proc Natl Acad Sci USA* **103:** 14877–14882

Black BE, Brock MA, Bedard S, Woods VLJ, Cleveland DW (2007) An epigenetic mark generated by the incorporation of CENP-A into centromeric nucleosomes. *Proc Natl Acad Sci USA* **104:** 5008–5013

Black BE, Foltz DR, Chakravarthy S, Luger K, Woods VL, Cleveland DW (2004) Structural determinants for generating centromeric chromatin. *Nature* **430:** 578–582

Blower MD, Karpen GH (2001) The role of *Drosophila* CID in kinetochore formation, cell-cycle progression and heterochromatin interactions. *Nat Cell Biol* **3:** 730–739

Blower MD, Sullivan BA, Karpen GH (2002) Conserved organization of centromeric chromatin in flies and humans. *Dev Cell* **2:** 319–330

Bouzinba-Segard H, Guais A, Francastel C (2006) Accumulation of small murine minor satellite transcripts leads to impaired centromeric architecture and function. *Proc Natl Acad Sci USA* **103:** 8709–8714

Buchwitz BJ, Ahmad K, Moore LL, Roth MB, Henikoff S (1999) A histone H3-like protein in *C. elegans*. *Nature* **401:** 547–548

Bühler M, Gasser SM (2009) Silent chromatin in the middle and at the end: lessons from yeasts. *EMBO J* (e-pub ahead of print 23 July 2009; doi:10.1038/emboj.2009.185)

Camahort R, Li B, Florens L, Swanson SK, Washburn MP (2007) Scm3 is essential to recruit the histone H3 variant CSE4 to centromeres and to maintain a functional kinetochore. *Mol Cell* **26:** 853–865

Carlson SR, Rudgers GW, Zieler H, Mach JM, Luo S, Grunden E, Krol C, Copenhaver GP, Preuss D (2007) Meiotic transmission of an *in vitro*-assembled autonomous maize minichromosome. *PLoS Genet* **3:** 1965–1974

Cheeseman IM, Niessen S, Anderson S, Hyndman F, Yates III JR, Oegema K, Desai A (2004) A conserved protein network controls assembly of the outer kinetochore and its ability to sustain tension. *Genes Dev* **18:** 2255–2268

Chen ES, Saitoh S, Yanagida M, Takahashi K (2003) A cell cycle-regulated GATA factor promotes centromeric localisation of CENP-A in fission yeast. *Mol Cell* **11:** 175–187

Chen Y, Baker RE, Keith KC, Harris K, Stoler S, Fitzgerald-Hayes M (2000) The N terminus of centromere H3-like protein Cse4p performs an essential function distinct from that of the histone fold domain. *Mol Cell Biol* **20:** 7037–7048

Choo KHA (2001) Domain organization at the centromere and neocentromere. *Dev Cell* **1:** 165–177

Clarke L, Carbon J (1980) Isolation of a yeast centromere and construction of functional small circular chromosomes. *Nature* **287:** 504–509

Collins KA, Furuyama S, Biggins S (2004) Proteolysis contributes to the exclusive centromere localization of the yeast Cse4/CENP-A histone H3 variant. *Curr Biol* **14:** 1968–1972

Conde e Silva N, Black BE, Sivolob A, Filipski J, Cleveland DW, Prunell A (2007) CENP-A-containing nucleosomes: easier disassembly versus exclusive centromeric localization. *J Mol Biol* **370:** 555–573

Dalal Y, Furuyama T, Vermaak D, Henikoff S (2007a) Structure, dynamics, and evolution of centromeric nucleosomes. *Proc Natl Acad Sci USA* **104:** 15974–15981

Dalal Y, Wang H, Lindsay S, Henikoff S (2007b) Tetrameric structure of centromeric nucleosomes in interphase *Drosophila* cells. *PLoS Biol* **5:** 1798–1809

Dunleavy EM, Pidoux AL, Monet M, Bonilla C, Richardson W, Hamilton GL, Ekwall K, McLaughlin PJ, Allshire RC (2007) A NASP (N1/N2)-related protein, Sim3, binds CENP-A and is required for its deposition at fission yeast centromeres. *Mol Cell* **28:** 1029–1044

Dunleavy EM, Roche D, Tagami H, Lacoste N, Ray-Gallet D, Nakamura Y, Daigo Y, Nakatani Y, Almouzni-Pettinotti G (2009) HJURP is a cell cycle dependent maintenance and deposition factor of CENP-A at centromeres. *Cell* **28:** 1029–1044

Earnshaw W, Migeon BR (1985) Three related centromere proteins are absent from the inactive centromere of a stable isodicentric chromosome. *Chromosoma* **92:** 290–295

Erhardt S, Mellone BG, Betts CM, Zhang W, Karpen GH, Straight AF (2008) Genome-wide analysis reveals a cell cycle-dependent mechanism controlling centromere propagation. *J Cell Biol* **183:** 805–818

Folco HD, Pidoux AL, Urano T, Allshire RC (2008) Heterochromatin and RNAi are required to establish CENP-A chromatin at centromeres. *Science* **319:** 94–97

Foltz DR, Jansen LET, Bailey AO, Yates III JR, Bassett EA, Wood S, Black BE, Cleveland DW (2009) Centromere specific assembly of CENP-A nucleosomes is mediated by HJURP. *Cell* **137:** 472–484

Foltz DR, Jansen LET, Black BE, Bailey AO, Yates III JR, Cleveland DW (2006) The human CENP-A centromeric nucleosome-associated complex. *Nature Cell Biol* **8:** 458–469

Fujita Y, Hayashi T, Kiyomitsu T, Toyoda Y, Kokubu A, Obuse C, Yanagida M (2007) Priming of centromere for CENP-A recruitment by human hMis18alpha, hMis18beta, and M18BP1. *Dev Cell* **12:** 17–30

Furuyama T, Dalal Y, Henikoff S (2006) Chaperone-mediated assembly of centromeric chromatin *in vitro*. *Proc Natl Acad Sci USA* **103:** 6172–6177

Glowczewski L, Yang P, Kalashnikova T, Santisteban MS, Smith MM (2000) Histone-histone interactions and centromere function. *Mol Cell Biol* **20:** 5700–5711

Goshima G, Kiyomitsu T, Yoda K, Yanagida M (2003) Human centromere chromatin protein hMis12, essential for equal segregation, is independent of CENP-A loading pathway. *J Cell Biol* **160:** 25–39

Goshima G, Saitoh S, Yanagida M (1999) Proper metaphase spindle length is determined by centromere proteins Mis12 and Mis6 required for faithful chromosome segregation. *Genes Dev* **13:** 1664–1677

Goshima G, Wollman R, Goodwin SS, Zhang N, Scholey JM, Vale RD, Stuurman N (2007) Genes required for mitotic spindle assembly in *Drosophila* S2 cells. *Science* **316:** 417–421

Hahnenberger KM, Baum MP, Polizzi CM, Carbon J, Clarke L (1989) Construction of functional artificial minichromosomes in the fission yeast *Schizosaccharomyces pombe*. *Proc Natl Acad Sci USA* **86:** 577–581

Harrington JJ, Van Bokkelen G, Mays RW, Gustashaw K, Willard HF (1997) Formation of *de novo* centromeres and construction of first-generation human artificial chromosomes. *Nature Genet* **15:** 345–355

Hayashi T, Fujita Y, Iwasaki O, Adachi Y, Takahashi K, Yanagida M (2004) Mis16 and Mis18 are required for CENP-A loading and histone deacetylation at centromeres. *Cell* **118:** 715–729

Hemmerich P, Weidtkamp-Peters S, Hoischen C, Schmiedeberg L, Erliandri I, Diekmann S (2008) Dynamics of inner kinetochore assembly and maintenance in living cells. *J Cell Biol* **180:** 1101–1114

Henikoff S, Ahmad K, Platero JS, van Steensel B (2000) Heterochromatic deposition of centromeric histone H3-like proteins. *Proc Natl Acad Sci USA* **97:** 716–721

Heun P, Erhardt S, Blower MD, Weiss S, Skora AD, Karpen GH (2006) Mislocalization of the *Drosophila* centromere-specific histone CID promotes formation of functional ectopic kinetochores. *Dev Cell* **10:** 303–315

Hori T, Amano M, Suzuki A, Backer CB, Welburn JP, Dong Y, McEwen BF, Shang WH, Suzuki E, Okawa K, Cheeseman IM, Fukagawa T (2008) CCAN makes multiple contacts with centromeric DNA to provide distinct pathways to the outer kinetochore. *Cell* **135:** 1039–1052

Howman EV, Fowler KJ, Newson AJ, Redward S, MacDonald AC, Kalitsis P, Choo KHA (2000) Early disruption of centromeric chromatin organization in centromere protein A (*Cenpa*) null mice. *Proc Natl Acad Sci USA* **97:** 1148–1153

Ishii K, Ogiyama Y, Chikashige Y, Soejima S, Masuda F, Kakuma T, Hiraoka Y, Takahashi K (2008) Heterochromatin integrity affects chromosome reorganization after centromere dysfunction. *Science* **321:** 1088–1091

Ito T, Tyler JK, Bulger M, Kobayashi R, Kadonaga JT (1996) ATP-facilitated chromatin assembly with a nucleoplasmin-like protein from *Drosophila melanogaster*. *J Biol Chem* **271:** 25041–25048

Jansen LET, Black BE, Foltz DR, Cleveland DW (2007) Propagation of centromeric chromatin requires exit from mitosis. *J Cell Biol* **176:** 795–805

Joglekar AP, Bouck DC, Molk JN, Bloom K, Salmon ED (2006) Molecular architecture of a kinetochore-microtubule attachment site. *Nature Cell Biol* **8:** 581–585

Keith KC, Baker RE, Chen Y, Harris K, Stoler S, Fitzgerald-Hayes M (1999) Analysis of primary structural determinants that distinguish the centromere-specific functions of histone variant Cse4p from histone H3. *Mol Cell Biol* **19:** 6130–6139

Ketel C, Wang HS, McClellan M, Bouchonville K, Selmecki A, Lahav T, Gerami-Nejad M, Berman J (2009) Neocentromeres form efficiently at multiple possible loci in *Candida albicans*. *PLoS Genet* **5:** e1000400

Kunitoku N, Sasamaya T, Marumoto T, Zhang D, Honda S, Kobayashi O, Hatakeyama K, Ushio Y, Saya H, Hirota T (2003) CENP-A phosphorylation by Aurora-A in prophase is required for enrichment of Aurora-B at inner centromeres and for kinetochore function. *Dev Cell* **5:** 853–864

Kwon MS, Hori T, Okada M, Fukagawa T (2007) CENP-C is involved in chromosome segregation, mitotic checkpoint function, and kinetochore assembly. *Mol Biol Cell* **18:** 2155–2168

Lermontova I, Schubert V, Fuchs J, Klatte S, Macas J, Schubert I (2006) Loading of *Arabidopsis* centromeric histone CENH3 occurs mainly during G2 and requires the presence of the histone fold domain. *Plant Cell* **18:** 2443–2451

Liu ST, Rattner JB, Jablonski SA, Yen TJ (2006) Mapping the assembly pathways that specify formation of the trilaminar kinetochore plates in human cells. *J Cell Biol* **175:** 41–53

Lo AW, Magliano DJ, Sibson MC, Kalitsis P, Craig JM, Choo KH (2001) A novel chromatin immunoprecipitation and array (CIA) analysis identifies a 460-kb CENP-A-binding neocentromere DNA. *Genome Res* **11:** 448–457

Maddox PS, Hyndman F, Monen J, Oegema K, Desai A (2007) Functional genomics identifies a Myb domain-containing protein family required for assembly of CENP-A chromatin. *J Cell Biol* **176:** 757–763

Malik HS, Henikoff S (2001) Adaptive evolution of Cid, a centromere-specific histone in *Drosophila*. *Genetics* **157:** 1293–1298

Marshall OJ, Chueh AC, Wong LH, Choo KH (2008) Neocentromeres: new insights into centromere structure, disease development and karyotype evolution. *Am J Hum Genet* **82:** 261–282

May BP, Lippman ZB, Fang Y, Spector DL, Martienssen RA (2005) Differential regulation of strand-specific transcripts from *Arabidopsis* centromeric satellite repeats. *PLoS Genet* **1:** e79

Measday V, Hailey DW, Pot I, Givan SA, Hyland KM, Cagney G, Fields S, Davis TN, Hieter P (2002) Ctf3p, the Mis6 budding yeast homolog, interacts with Mcm22p and Mcm16p at the yeast outer kinetochore. *Genes Dev* **16:** 101–113

Meluh PB, Yang P, Glowczewski L, Koshland D, Smith MM (1998) Cse4p is a component of the core centromere of *Saccharomyces cerevisiae*. *Cell* **94:** 607–613

Mizuguchi G, Xiao H, Wisniewski J, Smith MM, Wu C (2007) Nonhistone Scm3 and histones CenH3-H4 assemble the core of centromere-specific nucleosomes. *Cell* **129:** 1153–1164

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

Oegema K, Desai A, Rybina S, Kirkham M, Hyman AA (2001) Functional analysis of kinetochore assembly in *Caenorhabditis elegans*. *J Cell Biol* **153:** 1209–1226

Okada M, Cheeseman IM, Hori T, Okawa K, McLeod IX, Yates III JR, Desai A, Fukagawa T (2006) The CENP-H-I complex is required for the efficient incorporation of newly synthesized CENP-A into centromeres. *Nature Cell Biol* **8:** 446–457

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

Palmer DK, O'Day K, Trong HL, Charbonneau H, Margolis RL (1991) Purification of the centromere-specific protein CENP-A and demonstration that it is a distinctive histone. *Proc Natl Acad Sci USA* **88:** 3734–3738

Pearson CG, Yeh E, Gardner M, Odde D, Salmon ED, Bloom K (2004) Stable kinetochore-microtubule attachment constrains centromere positioning in metaphase. *Curr Biol* **14:** 1962–1967

Perpelescu M, Nozaki N, Obuse C, Yang H, Yoda K (2009) Active establishment of centromeric CENP-A chromatin by RSF complex. *J Cell Biol* **185:** 397–407

Pidoux AL, Choi ES, Abbott JKR, Liu X, Kagansky A, Castillo AG, Hamilton GL, Richardson W, Rappsilber J, He X, Allshire RC (2009) Fission yeast Scm3: a CENP-A receptor required for integrity of subkinetochore chromatin. *Mol Cell* **33:** 299–311

Pidoux AL, Richardson W, Allshire RC (2003) Sim4: a novel fission yeast kinetochore protein required for centromeric silencing and chromosome segregation. *J Cell Biol* **161:** 295–307

Polizzi C, Clarke L (1991) The chromatin structure of centromeres from fission yeast: differentiation of the central core correlates with function. *J Cell Biol* **112:** 191–201

Przewloka MR, Zhang W, Costa P, Archambault V, D'Avino PP, Lilley KS, Laue ED, McAinsh AD, Glover DM (2007) Molecular analysis of core kinetochore composition and assembly in *Drosophila melanogaster*. *PLoS ONE* **2:** e478

Régnier V, Vagnarelli P, Fukagawa T, Zerjal T, Burns E, Trouche D, Earnshaw W, Brown W (2005) CENP-A is required for accurate chromosome segregation and sustained kinetochore association of BubR1. *Mol Cell Biol* **25:** 3967–3981

Rieder CL (1979) Ribonucleoprotein staining of centrioles and kinetochores in newt lung cell spindles. *J Cell Biol* **80:** 1–9

Saffery R, Sumer H, Hassan S, Wong LH, Craig JM, Todokoro K, Anderson M, Stafford A, Choo KHA (2003) Transcription within a functional human centromere. *Mol Cell* **12:** 509–516

Santaguida S, Musacchio A (2009) The life and miracles of kinetochores. *EMBO J* (e-pub ahead of print 23 July 2009; doi:10.1038/emboj.2009.173)

Schuh M, Lehner CF, Heidmann S (2007) Incorporation of *Drosophila* CID/CENP-A and CENP-C into centromeres during early embryonic anaphase. *Curr Biol* **17:** 237–243

Sharp JA, Franco AA, Osley MA, Kaufman PD (2002) Chromatin assembly factor I and Hir proteins contribute to building functional kinetochores in *S. cerevisiae*. *Genes Dev* **16:** 85–100

Shelby RD, Monier K, Sullivan KF (2000) Chromatin assembly at kinetochores is uncoupled from DNA replication. *J Cell Biol* **151:** 1113–1118

Shelby RD, Vafa O, Sullivan KF (1997) Assembly of CENP-A into centromeric chromatin requires a cooperative array of nucleosomal DNA contact sites. *J Cell Biol* **136:** 501–513

Smith MM, Yang P, Santisteban MS, Boone PW, Goldstein AT, Megge PC (1996) A novel histone H4 mutant defective in nuclear division and mitotic chromosome transmission. *Mol Cell Biol* **16:** 1017–1026

Stoler S, Keith KC, Curnick KE, Fitzgerald-Hayes M (1995) A mutation in *CSE4*, an essential gene encoding a novel chromatin-associated protein in yeast, causes chromosome nondisjunction and cell cycle arrest at mitosis. *Genes Dev* **9:** 573–586

Stoler S, Rogers K, Weitze S, Morey L, Fitzgerald-Hayes M, Baker RE (2007) Scm3, an essential *Saccharomyces cerevisiae* centromere protein required for G2/M progression and Cse4 localization. *Proc Natl Acad Sci USA* **104:** 10571–10576

Sullivan BA, Blower MD, Karpen GH (2001) Determining centromere identity: cyclical stories and forking paths. *Nat Rev Genet* **2:** 584–596

Sullivan BA, Karpen GH (2004) Centromeric chromatin exhibits a histone modification pattern that is distinct from both euchromatin and heterochromatin. *Nature Struct Mol Biol* **11:** 1076–1083

Sullivan BA, Schwartz S (1995) Identification of centromeric antigens in dicentric Robertsonian translocations: CENP-C and CENP-E are necessary components of functional centromeres. *Hum Mol Genet* **4:** 2189–2197

Takahashi K, Chen ES, Yanagida M (2000) Requirement of Mis6 centromere connector for localizing a CENP-A-like protein in fission yeast. *Science* **288:** 2215–2219

Takahashi K, Murakami S, Shikashige Y, Funabiki H, Niwa O, Yanagida M (1992) A low copy number central sequence with strict symmetry and unusual chromatin structure in fission yeast centromere. *Mol Biol Cell* **3:** 819–835

Takayama Y, Sato H, Saitoh S, Ogiyama Y, Masuda F, Takahashi K (2008) Biphasic incorporation of centromeric histone CENP-A in fission yeast. *Mol Biol Cell* **19:** 682–690

Talbert PB, Bryson TD, Henikoff S (2004) Adaptive evolution of centromere proteins in plants and animals. *J Biol* **3:** 18

Talbert PB, Masuelli R, Tyagi AP, Comai L, Henikoff S (2002) Centromeric localization and adaptive evolution of an *Arabidopsis* histone H3 variant. *Plant Cell* **14:** 1053–1066

Topp CN, Zhong CX, Dawe RK (2004) Centromere-encoded RNAs are integral components of the maize kinetochore. *Proc Natl Acad Sci USA* **101:** 15986–15991

Van Hooser AA, Ouspenski II, Gregson HC, Starr DA, Yen TJ, Goldberg ML, Yokomori K, Earnshaw WC, Sullivan KF, Brinkley BR (2001) Specification of kinetochore-forming chromatin by the histone H3 variant CENP-A. *J Cell Sci* **114:** 3529–3542

Vermaak D, Hayden HS, Henikoff S (2002) Centromere targeting element within the histone fold domain of Cid. *Mol Cell Biol* **22:** 7553–7561

Walfridsson J, Bjerling P, Thalen M, Yoo EJ, Park SD, Ekwall K (2005) The CHD remodeling factor Hrp1 stimulates CENP-A loading to centromeres. *Nucleic Acids Res* **33:** 2868–2879

Warburton PE (2004) Chromosomal dynamics of human neocentromere formation. *Chromosome Res* **12:** 617–626

Warburton PE, Cooke CA, Bourassa S, Vafa O, Sullivan BA, Stetten G, Gimelli G, Warburton D, Tyler-Smith C, Sullivan KF, Poirier GG, Earnshaw WC (1997) Immunolocalization of CENP-A suggests a distinct nucleosome structure at the inner kinetochore plate of active centromeres. *Curr Biol* **7:** 901–904

Williams BC, Murphy TD, Goldberg ML, Karpen GH (1998) Neocentromere activity of structurally acentric mini-chromosomes in *Drosophila*. *Nature Genet* **18:** 30–37

Williams JS, Hayashi T, Yanagida M, Russell P (2009) Fission yeast Scm3 mediates stable assembly of Cnp1/CENP-A into centromeric chromatin. *Mol Cell* **33:** 287–298

Yang CH, Tomkiel J, Saitoh H, Johnson DH, Earnshaw WC (1996) Identification of overlapping DNA-binding and centromere-targeting domains in the human kinetochore protein CENP-C. *Mol Cell Biol* **16:** 3576–3586

Yoda K, Ando S, Morishita S, Houmura K, Hashimoto K, Takeyasu K, Okazaki T (2000) Human centromere protein A (CENP-A) can replace histone H3 in nucleosome reconstitution *in vitro*. *Proc Natl Acad Sci USA* **97:** 7266–7271

Zeitlin SG, Shelby RD, Sullivan KF (2001) CENP-A is phosphorylated by Aurora B kinase and plays an unexpected role in completion of cytokinesis. *J Cell Biol* **155:** 1147–1157

