Two distinct pathways responsible for the loading of CENP-A to centromeres in the fission yeast cell cycle

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CENP-A is a centromere-specific histone H3 variant that is essential for faithful chromosome segregation in all eukaryotes thus far investigated. We genetically identified two factors, Ams2 and Mis6, each of which is required for the correct centromere localization of SpCENP-A (Cnp1), the fission yeast homologue of CENP-A. Ams2 is a cell-cycle-regulated GATA factor that localizes on the nuclear chromatin, including on centromeres, during the S phase. Ams2 may be responsible for the replication-coupled loading of SpCENP-A by facilitating nucleosomal formation during the S phase. Consistently, overproduction of histone H4, but not that of H3, suppressed the defect of SpCENP-A localization in Ams2-deficient cells. We demonstrated the existence of at least two distinct phases for SpCENP-A loading during the cell cycle: the S phase and the late-G2 phase. Ectopically induced SpCENP-A was efficiently loaded onto the centromeres in G2-arrested cells, indicating that SpCENP-A probably undergoes replication-uncoupled loading after the completion of S phase. This G2 loading pathway of SpCENP-A may require Mis6, a constitutive centromere-binding protein that is also implicated in the Mad2-dependent spindle attachment checkpoint response. Here, we discuss the functional relationship between the flexible loading mechanism of CENP-A and the plasticity of centromere chromatin formation in fission yeast.

Keywords: CENP-A histone; centromere; chromosome segregation; GATA factor; fission yeast

1. INTRODUCTION

The kinetochore is a multiprotein–DNA complex that mediates spindle microtubule attachment during the M phase and, therefore, it is essential for the poleward movement of chromosomes in both mitosis and meiosis (Cleveland *et al.* 2003). To adjust the microtubule-driven dynamic behaviour of chromosomes with the progression of the M phase, the inter-kinetochore tension and the outer-kinetochore association with microtubules are thought to be sensed by the BubR1–Bub1-dependent and the Mad2–Mad1-dependent mitotic checkpoint mechanisms, respectively (Skoufias *et al.* 2001). The kinetochores play a central role in mitotic checkpoint regulation as signal generating factories that control the cell cycle progression until all chromosomes are properly captured by bipolar spindles (Cleveland *et al.* 2003). In order to prevent chromosomal breakage, the kinetochore assembly must be restricted on a single locus, the centromere, in each chromosome. Surprisingly, despite this restriction, in most eukaryotes, the centromere is not defined by a consensus DNA sequence, but rather shows an adaptable feature as regards its location on the

chromosome (Karpen & Allshire 1997; Cleveland *et al.* 2003). A typical example of the plasticity of centromere chromatin is neocentromere formation (figure 1a). The neocentromere is an ectopic centromere formed *de novo* at a hitherto noncentromeric chromosomal location when authentic centromeres are eventually deleted. This occasional ‘rescue’ event from a crisis in which an acentric chromosome undergoes at the M phase has been documented in human tissue culture cells, and a number of neo centromere hot spots have been mapped on chromosomes (Choo 2001). Although this centromere repositioning has been proposed to provide a potentially powerful evolutionary force for reproductive isolation and speciation (Henikoff *et al.* 2001; Amor & Choo 2002), the underlying molecular mechanisms of this repositioning remain ill-defined. Another process that is demonstrative of the flexibility of centromere formation is a phenomenon referred to as ‘centromere inactivation’ (figure 1b). In human tissue culture cells, stable dicentric chromosomes have been found in which one centromere has been inactivated and thus becomes unable to associate with spindle microtubules (Sullivan & Willard 1998). This example clearly indicates that the centromeric DNA sequence alone is not able to determine centromere functions. Once the centromere chromatin is established at one site on the chromosome, the potential centromere activities at other loci may be stably silenced. Recently, a human family was described in which centromere

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Figure 1. Examples of neo centromere formation (a) and centromere inactivation (b). (i) Rare internal recombination has produced a ring chromosome and a linear deletion chromosome that lost the authentic centromere. (ii) The acentric chromosome would not be correctly propagated into the daughter cells owing to the failure of the association with mitotic spindles (hashed circles and black lines represent centrosomes and microtubules, respectively) during the M phase. (iii) If a neo centromere originates from a non-centromeric arm region before a cell passes through the subsequent M phase, the cell can survive a crisis at the M phase. (iv) An occasional chromosomal end fusion between two chromatids would produce a deleterious dicentric chromosome. (v) Two active centromeres (closed circles) on the dicentric chromosome may lead to chromosomal breakage in anaphase. (vi) If one of two centromeres on the dicentric chromosome has been stably inactivated (an open circle), a cell could potentially avoid chromosomal breakage in the M phase.

repositioning occurred on chromosome 4 via neocentromere formation with the retention of α -satellite DNA, but without any other detectable chromosome alterations (Amor *et al.* 2004). This pseudodicentric-neocentric chromosome was shown to be stably transmitted, both in mitosis and meiosis. The ability of the neocentric activity to form at a euchromatic site rather than at a pre-existing alphoid domain provides direct evidence for an inherent mechanism responsible for human centromere repositioning and karyotype evolution. This strong epigenetic inactivation of an original centromere may indicate the existence of chromatin-based inhibition of the generation of other active centromeres other than the centromere presently in use.

The DNA sequences in the centromeric loci bear little resemblance among diverse species (Henikoff *et al.* 2001). In all eukaryotes examined to date, a hallmark of functional centromeres is the accumulation of CENP-A, a centromere-specific histone H3 variant (Palmer *et al.* 1987, 1991; Smith 2002). From that in budding yeast carrying non-repetitive 'point' centromeres to that in holocentric Nematoda, CENP-A has been shown to replace histone H3 in centromere nucleosomes, and it is known to be essential for proper chromosome segregation (Stoler *et al.* 1995; Meluh *et al.* 1998; Buchwitz *et al.* 1999; Howman *et al.* 2000; Takahashi *et al.* 2000; Blower & Karpen 2001; Goshima *et al.* 2003). Interestingly, CENP-A has

been shown to be localized at active, but not at inactive, centromeres in stable dicentric chromosomes (Earnshaw & Migeon 1985; Warburton *et al.* 1997; Sullivan & Willard 1998). Therefore, CENP-A is thought to be a determinant of centromere identity (Henikoff *et al.* 2001; Black *et al.* 2004). One attractive hypothesis is that a rapid change in CENP-A localization, based on the flexibility of CENP-A reloading, may enable the centromere chromatin to be repositioned and re-established at a given time, that is, within interphase before subsequently entering the M phase.

To investigate the loading mechanism of CENP-A using molecular genetics, the fission yeast, *Schizosaccharomyces pombe*, is an ideal organism because its centromere structure shares features in common with those in metazoans (Sullivan *et al.* 2001), and a number of mutants defective in chromosome dynamics and cell cycle regulation are available (Egel 2004). Fission yeast has three chromosomes with complex centromeres that occupy between approximately 35 and 110 kb in length (Chikashige *et al.* 1989; Murakami *et al.* 1991). The nucleotide sequences of entire regions have been determined (Takahashi *et al.* 1992). The three centromeres (*cen1*, *cen2* and *cen3*) are organized in a similar fashion, with a central core region flanked by outer repeat regions; both regions are required for ensuring mitotic and meiotic centromere functions (Chikashige *et al.* 1989; Niwa *et al.* 1989; Clarke & Baum 1990; Matsumoto *et al.* 1990; Hahnenberger *et al.* 1991; Murakami *et al.* 1991; Takahashi *et al.* 1992; Baum *et al.* 1994; Watanabe & Nurse 1999). An outer repeat containing repetitive motifs common to all centromeres (Nakaseko *et al.* 1986, 1987; Murakami *et al.* 1991) forms the pericentric heterochromatin, which is responsible for the sister-centromere cohesion (Partridge *et al.* 2000; Bernard *et al.* 2001), whereas a ca. 15 kb long central core forms a bona fide kinetochore with the specialized chromatin (figure 2a; Polizzi & Clarke 1991; Takahashi *et al.* 1992) and probably confers the bioriented nature to the centromeres (Matsumoto *et al.* 1990; Saitoh *et al.* 1997; Goshima *et al.* 1999; Takahashi *et al.* 2000). To date, a number of evolutionarily conserved kinetochore and heterochromatin proteins have been identified, which have been shown to bind to the central core and outer repeat regions, respectively (Allshire 2004).

Different centromeric states have been reported in fission yeast. Minichromosomes containing only a portion of the centromeric DNA adopt either a mitotically stable or a mitotically unstable state. A mitotically unstable centromere switches to the stable state at a frequency of 0.6–0.7% without any DNA rearrangements, suggesting that two heritable states of the centromere chromatin could be formed in fission yeast wild-type cells (Steiner & Clarke 1994). Specific inhibition of histone deacetylation activity using Trichostatin A (TSA) has been shown to compromise centromere integrity, leading to the mislocalization of heterochromatin proteins and partial defects in chromosome segregation in fission yeast (Ekwall *et al.* 1997). After the removal of TSA, the hyperacetylation state of histones at the centromere,

Figure 2. SpCENP-A is required for the formation of the centromere chromatin that ensures equal chromosome segregation in the M phase. (a) Nuclear chromatin was prepared from germinated wild-type cells (wt) and SpCENP-A disruptant cells (null) that were digested with MNase for 0, 1, 2, 4 and 8 min, followed by Southern blot analysis with the three centromeric DNA probes otr, imr and cnt. An arrowhead indicates the position of mono-nucleosome. (b) Shown at left is the germination phenotype of SpCENP-A-null cells with large and small daughter nuclei (arrowheads) stained by the DNA-specific stain DAPI. Shown at right are representative SpCENP-A-null cells displaying the separated cen1-GFP in one or two unequally sized nuclei. Scale bar, 10 µm. Taken from Takahashi *et al.* (2000).

but not at non-centromeric arm regions, was epigenetically propagated to subsequent generations. The TSA-induced state exhibiting the partial centromere inactivation was indeed transmitted within several rounds of generations. These observations indicate that the chromatin-based, adaptable mechanisms in centromere formation do in fact function in fission yeast cells. In this report, we describe the results of our recent attempts to uncover the relationships between the plasticity of centromere chromatin formation and the flexible loading mechanisms of CENP-A using fission yeast as a model organism.

2. FISSION YEAST CENP-A IS REQUIRED FOR THE FORMATION OF CENTROMERE-SPECIFIC CHROMATIN

To address the possible role of CENP-A loading in centromere formation and function, a gene encoding an *S. pombe* CENP-A homologue (SpCENP-A; the gene name is designated as *cnp1*⁺) was identified and characterized (Takahashi *et al.* 2000). We have shown in a previous study that SpCENP-A is a constitutive centromere protein that is essential for equal chromosome segregation in mitosis. Our chromatin immunoprecipitation (ChIP) assay revealed that SpCENP-A is specifically localized into the central core region of centromeres. This central centromeric DNA (cnt: centre and imr: inmost repeat; Takahashi *et al.* 1992)

has been shown to contain specialized chromatin that yields a smear micrococcal nuclease (MNase) digestion pattern, whereas the outer repetitive regions (otr: outer repetitive; Takahashi *et al.* 1992) exhibited a regular pattern of nucleosomes (Polizzi & Clarke, 1991; Takahashi *et al.* 1992). In SpCENP-A-deficient cells, the smear pattern was abolished, thus indicating that SpCENP-A is essential for centromere-specific chromatin formation (figure 2a; Takahashi *et al.* 2000). Even in the absence of SpCENP-A, the central core regions still exhibited the pattern of a normal nucleosomal ladder, indicating that the histone H3 was presumably incorporated into the centromere nucleosomes in this mutant. These H3-containing centromeres lost the bioriented nature, producing unequal-sized nuclei during mitosis (figure 2b, left-hand side; Takahashi *et al.* 2000). Cytokinesis occurred after unequal nuclear division, leading to aneuploidy. To elucidate the dynamics of the centromeres, we used a cen1-GFP probe (Nabeshima *et al.* 1998) in the disruptant (figure 2b, right-hand side). In some cells, two separate GFP signals were detected within one daughter nucleus, indicating that the missegregation of chromosome I actually took place in this case. The remaining binucleate cells had normally distributed cen1-GFP signals, but most nuclei were unequal in size, suggesting missegregation took place in other chromosomes. Therefore, it is likely that the centromeres still possess the capability of interacting with mitotic spindles and of moving toward the spindle poles in anaphase just after the depletion of SpCENP-A. These results indicate that SpCENP-A-containing nucleosomes are primarily required for the formation of bioriented centromere chromatin, which in turn ensures equal chromosome segregation during the M phase.

We next created a recessive ts allele of the SpCENP-A (*cnp1*⁺) gene, *cnp1-1*, and we demonstrated that *cnp1-1* ts cells exhibited a phenotype identical to that of SpCENP-A-null cells, with decreased viability after unequal mitotic segregation at a restricted temperature (36 °C; figure 3a; Takahashi *et al.* 2000). Using this ts strain, we examined the 'recovery' step of the fission yeast centromere chromatin during the cell cycle. We performed an MNase digestion experiment with a synchronous G1 population of *cnp1-1* ts mutant cells (figure 3b; Takahashi *et al.* 2000). *cnp1-1* cells were arrested at the G1 phase by nitrogen starvation and were then released into the complete medium at 36 °C. The cell cycle progression was monitored by measuring the DNA contents (figure 3b, left-hand side), cell viability and missegregation frequency (figure 3b, centre). The smear pattern of centromere chromatin was observed in both G1-arrested and exponentially growing wild-type cells. In contrast, the smear pattern of *cnp1-1* mutant cells was already partly reduced at a permissive temperature (20 °C) and clearly lost at 6 h in 36 °C (figure 3b, right-hand side). In the ts mutant, the frequency of missegregation increased only after 8 h, and became prominent after 10 h at 36 °C, resulting in a decrease in cell viability. Thus, the disruption of the centromere chromatin occurred before chromosome missegregation in SpCENP-A-deficient cells. The cell

Figure 3. The disruption of the centromere-specific chromatin before unequal chromosome segregation in SpCENP-A *ts* cells. (a) The phenotype of SpCENP-A *ts* (*cnp1-1*) mutant cells cultured in YPD at 20 or 36 °C for 6 h (top). Cell viability (%) and frequency (%) of binucleate cells showing the asymmetric nuclei of the *ts* mutant cells (bottom). (b) Wild-type and *cnp1-1 ts* cells in the G1 phase were prepared by nitrogen starvation at 20 °C and were synchronously released at 36 °C. Shown from left to right are the DNA contents estimated by flow cytometry, cell viability, frequency of binucleate cells with asymmetric nuclei and MNase digestion experiments with *otr* and *imr* probes for Southern hybridization. (c) Intracellular localization and the level of SpCENP-A^{ts} (Cnp1-1) mutant protein at 20 or 33 °C. Wild-type cells expressing the tagged SpCENP-A^{ts}-GFP or SpCENP-A-GFP integrated at the *lys1* locus were cultured in EMM2 at 20 °C; the temperature was shifted to 33 °C for 5 h, and then was returned again to 20 °C. Upper panel: localization of GFP signals. Lower panel: the protein levels of GFP-tagged wild-type (SpCENP-A-GFP) and *ts* SpCENP-A (SpCENP-A^{ts}-GFP). The localizations of GFP-tagged proteins are presented at the indicated time points. Note that the doubling time of the cells was approximately 3 h at 33 °C and 10 h at 20 °C in this culture medium. Cdc2 kinase (antibodies to PSTAIRE) was used as a loading control. Bar, 10 µm. Taken from Takahashi *et al.* (2000) and Chen *et al.* (2003b).

viability of the *ts* mutant remained intact at 6 h at 36 °C, suggesting that the disrupted centromere chromatin could be re-established before entering the subsequent M phase. This re-establishment of the centromere chromatin probably takes place rapidly, even at the late G2 phase, implying that the fission yeast centromere has flexible properties, that is, the disrupted bioriented chromatin structure can be repaired in a cell-cycle-independent manner. In other words, continuous incubation of *cnp1-1* mutants throughout one round of the cell cycle is necessary to retain the disruption of the centromere-specific chromatin, which, in turn, results in chromosome missegregation in the following M phase. In genes encoding other central core binding proteins, not only *cnp1-1* but also *ts* mutations such as *mis6-302* and *mis12-537*, have been shown to exhibit the same phenotypic features (Saitoh *et al.* 1997; Goshima *et al.* 1999; Takahashi *et al.* 2000; Hayashi *et al.* 2004). Such findings indicate that the formation or reformation of the fission yeast centromere-specific chromatin would be related to the basic system of

repair that ensures the quick assembly of centromere core complexes in case of emergency.

As an initial attempt to elucidate the rapid recovery mechanisms of the centromere chromatin structure in *cnp1-1* mutant cells, we observed the behaviour of SpCENP-A *ts* protein (SpCENP-A^{ts}) at both permissive (20 °C) and restrictive temperatures (33–36 °C) in a wild-type background. To this end, a wild-type strain bearing the SpCENP-A^{ts}-GFP gene extragenically integrated onto the *lys1* locus was constructed. The centromere localization of SpCENP-A^{ts}-GFP protein was found to be normal at 20 °C (figure 3c; Chen *et al.* 2003a,b). After raising the temperature to 33 °C, the GFP signal of the mutant protein at the centromere was abolished. The mutant protein level, determined by immunoblot analysis using anti-GFP antibodies, slightly decreased at 33 °C (figure 3c, lower panel). Upon returning to 20 °C, the mislocated SpCENP-A^{ts}-GFP had again accumulated, as observed by centromeric dot signals, thus indicating that the ability of SpCENP-A^{ts} to localize at the centromere is

Figure 4. Factors responsible for the centromeric localization of SpCENP-A or Mad2. Ams2 is a cell-cycle-regulated GATA-type transcription factor that ensures the replication-coupled incorporation of SpCENP-A and facilitates nucleosomal formation by activating histone gene transcription (i; Chen *et al.* 2003a,b; Takayama & Takahashi, in preparation). The Mis6 central core complex is a constitutive centromere subcomplex that is essential for SpCENP-A localization at the centromeres throughout the cell cycle (ii; Saitoh *et al.* 1997; Takahashi *et al.* 2000; Pidoux *et al.* 2004; Hayashi *et al.* 2004). The Mis6-Sim4 complex is required for the correct accumulation of Mad2 at centromeres that have not yet attached to the spindle microtubules during early M phase (v); the Nuf2-complex is also required at this point (vi; Saitoh & Takahashi, in preparation). Mis16 shows high homology with human retinoblastoma-associated proteins, RbAp48 and RbAp46, and forms a complex with Mis18 (Hayashi *et al.* 2004). The Mis16-Mis18 complex is required for the association of the Mis6-complex (iv) and SpCENP-A (iii) with the centromeres and plays an important role in maintaining the level of deacetylated histones within the central core regions of centromeres (Hayashi *et al.* 2004).

temperature-sensitive. The level of mutant SpCENP-A^{ts} protein was also restored 1 h after the temperature shift to 20 °C. The recovery of GFP signals occurred rapidly within 0.5–1 h after the shift to 20 °C (the doubling time of the cells under this experimental condition was approximately 10 h). Therefore, the recovery of SpCENP-A^{ts} protein to the centromeres was most likely to have proceeded efficiently, even after the cells had completed the S phase (exponentially growing *S. pombe* cells were mostly in the G2 phase). The above observation suggests that the flexible reloading of SpCENP-A into the centromere nucleosomes could be one of the molecular bases for the rapid regeneration of the impaired centromere chromatin structure.

3. FACTORS RESPONSIBLE FOR CENP-A LOADING IN FISSION YEAST

In order to gain a better understanding of how SpCENP-A is deposited in the pre-existing or newly defined centromere locus, we have tried to identify the loading factors for SpCENP-A using a couple of genetic approaches. So far, researchers have identified at least three different classes of component responsible for correct SpCENP-A loading onto the centromeres: the Mis6-Sim4 complex, the Mis16-Mis18 complex and the Ams2 GATA factor (figure 4).

Mis6 was originally identified as a *mis* (minichromosome instability) gene product, the *ts* mutation of which causes frequent minichromosome loss at the permissive temperature and fatal unequal chromosome segregation at the restrictive temperature (Takahashi *et al.* 1994). Mis6 constitutively binds to the central core regions of centromeres (Saitoh *et al.* 1997) and forms a centromere subcomplex with Sim4 (Pidoux *et al.* 2004), Mis15 and Mis17 (Hayashi *et al.* 2004). Depletion of each component of the Mis6 subcomplex was found to result in the significant reduction of SpCENP-A localization at the centromeres and also led to the disruption of the centromere-specific chromatin (Takahashi *et al.* 2000; Pidoux *et al.* 2003; Hayashi *et al.* 2004). Mis6 and Sim4 have human homologues, hMis6/CENP-I and CENP-H, respectively (Saitoh *et al.* 1997; Nishihashi *et al.* 2002; Goshima *et al.* 2003; Pidoux *et al.* 2003). Recently, we demonstrated that the Mis6-Sim4 complex is also required for the Mad2-related spindle attachment checkpoint response (our unpublished results): in Mis6 or Sim4-deficient cells, the Mad2 but not the Bub1 spindle checkpoint protein failed to accumulate on centromeres that had not attached to the spindle microtubules during the early M phase. The Nuf2-Hec1 centromere complex is another essential component of the Mad2 loading pathway (Saitoh & Takahashi, in preparation). In human cells, hMis6/CENP-I and the hNuf2-hHec1 complex have been also shown to be required for the kinetochore accumulation of hMad2 and hMad1, but not for that of hBubR1 and hBub1 (Martin-Lluesma *et al.* 2002; DeLuca *et al.* 2003; Liu *et al.* 2003). The roles of these complexes in Mad2 localization appear to be evolutionarily conserved. In fission yeast, the Mis6-Sim4 complex plays dual roles in both SpCENP-A incorporation into the centromere nucleosomes and Mad2 accumulation on the surface of unattached kinetochores. These findings imply that the Mis6-Sim4 complex acts as a molecular interface that potentially transmits the positional information regarding the spindle attachment site to the SpCENP-A loading pathway (Saitoh & Takahashi, in preparation).

Mis16 and Mis18 bind to the central centromeres and form a complex that is essential for an evolutionarily conserved CENP-A loading pathway (Hayashi *et al.* 2004). Interestingly, in these mutant cells, a marked increase in the level of histone H3 and H4 acetylation was observed in the central centromeres (Hayashi *et al.* 2004). Mis16 and Mis18 are the most upstream factors in kinetochore assembly because they are able to associate with kinetochores in all kinetochore mutants except for *mis18* and *mis16*, respectively. Conversely, the Mis16-Mis18 complex is required for the centromeric localization of the Mis6-Mis15-Mis17 complex and SpCENP-A. *mis16*⁺ encodes an evolutionarily conserved WD repeat-containing protein that shows similarity to human RbAp46 and RbAp48 (50–53% identity; Hayashi *et al.* 2004). In fission yeast, Prw1, a component of the Clr6-containing histone deacetylase complex (Nakayama *et al.* 2003), exhibits 44% identity with Mis16, although Prw1 and Mis16 most probably execute distinct functions (Hayashi *et al.* 2004). RbAp46/48 was found to be a ubiquitous binding

Figure 5. Ams2 is required for the selective incorporation of SpCENP-A into the centromere nucleosomes, but is not required for the centromere localization of other central core proteins. (a) SpCENP-A-HA expressed in either Δ ams2 or wild-type cells cultured in YES at 26 °C was immunoprecipitated (IP) for a ChIP analysis using antibodies against HA (α -HA) and the C-terminal region of human histone H3 (α -CENP-A+H3). This antibody recognizes both SpCENP-A and histone H3 in fission yeast (data not shown). Co-precipitated DNAs were amplified by PCR using the central centromere (cnt1, imr1) and arm region (lys1) primers (Takahashi *et al.* 2000). Lane 5 is a control lane carried out with beads alone. The intensity of each IP signal was divided by that of the corresponding whole cell extract (WCE) signal after the background titration, and shown in the right panel as the relative intensity (taken from Chen *et al.* 2003a). (b) Localization of SpCENP-A-GFP, Mis6-GFP, or Mis12-GFP in wild-type and Δ ams2 cells cultured in EMM2 at 26 °C. The percentages of cells showing proper centromeric (grey), weak centromeric (striped) or dispersed nuclear (white) patterns are shown in the bottom panel (taken from Chen *et al.* 2003a).

partner of retinoblastoma (Rb) tumour suppressor protein (Qian *et al.* 1993; Qian & Lee 1995) and a component of the human chromatin-assembly factor-1 (CAF-1) complex that promotes the assembly of nucleosomes onto newly replicated DNA (Verreault *et al.* 1996; Krude 1999). In wild-type cells, the central centromeric nucleosomes consist of highly deacetylated histones, a state which is maintained through Mis16–Mis18 functions (Hayashi *et al.* 2004). Hypoacetylated state of the centromeric histones may be a prerequisite for the correct loading of SpCENP-A.

The third component of the SpCENP-A loading pathways is Ams2, a cell-cycle-regulated GATA-type transcription factor (Chen *et al.* 2003a,b). Ams2 was originally identified as a gene product of a multicopy suppressor of the *cnp1-1* ts mutant. Ams2 is one of four GATA factors found in *S. pombe*, containing a zinc finger DNA binding motif with two pairs of cysteine residues, a characteristic feature of the GATA factors. Despite a significant reduction in SpCENP-A localization at the centromeres in Ams2-null cells, Ams2 is not essential for cell viability (Chen *et al.* 2003a). This means that Ams2 sharply contrast with other components of SpCENP-A loading pathways, which are all essential, and the depletion of which leads to a terminal

phenotype of cells as same as that of SpCENP-A-null cells.

Therefore, an obvious question in this context would be how such a non-essential *ams2*⁺ gene product is able to exert an effect on essential SpCENP-A localization. The Ams2 disruptant shows growth retardation with chromosome missegregation (Chen *et al.* 2003a). Although FISH analyses revealed that only 6% of the population of mitotic Δ ams2 cells showed missegregation of chromosome III, a chromatin IP experiment revealed that the amount of SpCENP-A bound to the central centromeric DNAs was significantly reduced in Ams2-deficient cells, as compared with that in wild-type cells (figure 5a; Chen *et al.* 2003a). In Δ ams2 cells, the total amount of SpCENP-A binding at the central centromeres was reproducibly two- to fourfold (cnt) and four- to tenfold (imr) less than that observed in the wild-type controls (lanes 1 and 3). A ChIP experiment using anti-human H3 antibodies was also carried out using the same cell extracts. The antigen for this antibody contains an amino acid sequence that is not only identical to that of the *S. pombe* histone H3 C-terminal, but is also very similar to that of the SpCENP-A C-terminal. This antibody was indeed shown to recognize immunoprecipitated SpCENP-A, as well as histone H3 (data not

shown). Thus, the strength of the PCR signal amplified using this antibody (α CENP-A+H3) is likely to represent both SpCENP-A and histone H3 binding. In Δ ams2 cells, the total amount of SpCENP-A and histone H3 binding at the central centromeres was greater than that of wild-type cells (lanes 2 and 4), despite the decrease in SpCENP-A binding (lanes 1 and 3). Note that such differences were not observed at the *lys1* locus (lower right). These findings suggest that the decrease in SpCENP-A was compensated for, at least partly, by histone H3 within the central centromeres in Ams2-deficient cells.

Surprisingly, both Mis6 and Mis12 are centromerically localized correctly in Δ ams2 cells, indicating that a loss of Ams2 function clearly affects the centromere localization of SpCENP-A but not that of other centromere proteins (figure 5b; Chen *et al.* 2003a). These results suggest that, in Δ ams2 cells, the significant population of H3-containing centromere nucleosomes still probably possess the ability to bind to other central core proteins. This conclusion is seemingly inconsistent with a notion proposed in the case of other organisms, that is, that CENP-A is the most fundamental building block for the kinetochore architecture (Henikoff *et al.* 2001). However, we noted that roughly 20% of Δ ams2 cells showed GFP signals at the centromeres that were as bright as those observed in wild-type cells (figure 5b, lower panel; Chen *et al.* 2003a). In particular, binucleated cells tended to be GFP-positive in Δ ams2 cells. In the absence of Ams2, SpCENP-A localization at the centromeres may, therefore, become cell-cycle-dependent, which may account for the retention of the central core proteins in Δ ams2 cells.

4. A REPLICATION-UNCOUPLLED LOADING ACTIVITY OF CENP-A DURING THE G2 PHASE

To observe the behaviour of SpCENP-A during the cell cycle, we performed live cell analyses using Δ ams2 cells carrying the native promoter-driven SpCENP-A-GFP gene. We found that the SpCENP-A-GFP signal is actually turned on and off during the cell cycle (Takayama & Takahashi, in preparation). At the mid-G2 phase, SpCENP-A-GFP signals accumulated gradually at the centromeres, and the intensity of these SpCENP-A-GFP signals peaked in the late G2 and in the M phase. After the cell division, the signals suddenly disappeared from the centromeres (Takayama & Takahashi, in preparation). Therefore, Ams2 appears to be required for the proper centromeric localization of SpCENP-A from the S or early-G2 to mid-G2 phase (figure 6a). We have demonstrated that Ams2 is cell cycle-regulated at the protein level, is localized at the nuclear chromatin around the G1/S phase and then rapidly disappears after cells begin to grow (figure 6c; Chen *et al.* 2003a). It is possible that Ams2 promotes SpCENP-A loading during the S phase and, therefore, Ams2 deletion might result in the sudden decrease in SpCENP-A-GFP signals at the early G2 phase. This assumption may be consistent with our recent data indicating that Ams2 is

Figure 6. Schematic of the intensity of SpCENP-A signals on the centromeres (a) and the transcriptional level of histone genes (H2A, H2B, H3 and H4) (b) in wild-type (dotted line) and Δ ams2 (solid line) cells during the cell cycle (our unpublished results). Two arrows indicate the distinct loading points of SpCENP-A in the S phase (i) (Ams2-dependent) and late-G2 phase (ii) (Ams2-independent) during the cell cycle, respectively. (c) Wild-type cells carrying the integrated Ams2-GFP were cultured in EMM2 at 26 °C. Cells at different stages of the cell cycle demonstrated striking cell cycle-dependent changes in localization. Ams2-GFP was enriched in the nucleus during the G1/S phase. The data regarding Ams2 localization were taken from Chen *et al.* (2003a).

required for the transcriptional activation of histone genes during the S phase (figure 6b; Takayama & Takahashi, in preparation). The Ams2 protein levels oscillated during the cell cycle and peaked at the same time as histone gene activation at the S phase. Using an *ams2* conditional-shut off strain, we successfully prepared a synchronous culture of Ams2-deficient cells. Northern blot analyses revealed that, although the basal transcription of histone genes was detected, the cell-cycle-dependent accumulation of histone mRNAs (H2A, H2B, H3 and H4) was totally diminished in Δ ams2 cells (Takayama & Takahashi, in preparation). In wild-type cells, the histone genes are activated at the S phase, and SpCENP-A is constitutively localized at the centromeres. However, in Δ ams2 cells, the histone genes are not activated, and SpCENP-A disappears just after the completion of the S phase (figure 6). In the absence of Ams2, the cell loses one of the main periodicities of the cell cycle; namely, the transcriptional oscillation of histone genes, and the resulting lack of free histones in the cells may, in turn, lead to a reduction in the efficiency of SpCENP-A incorporation into the centromere nucleosomes at the S phase.

Consistent with this hypothesis, we found that the supply of histone H4 (a binding partner of SpCENP-A) by a multicopy plasmid suppressed the growth retardation of Δ ams2 cells, whereas the overproduction of histone H3 (a competitor of SpCENP-A for H4 binding) was rather toxic to the growth of Δ ams2 cells (figure 7, left-hand side). The right panels of figure 7 show the effects of histone overproduction on SpCENP-A localization in the absence of Ams2.

Figure 7. Effects of the overproduction of histone H3 or H4 in *Ams2*-null cells on colony formation and SpCENP-A localization at the centromeres. Growth retardation of Δ *ams2* cells was partly suppressed by a multicopy plasmid carrying either the SpCENP-A (data not shown; Chen *et al.* 2003a) or the histone H4 gene, but growth retardation was further enhanced by a multicopy plasmid carrying the histone H3 gene. Shown in the columns at left are colonies of Δ *ams2* cells with the indicated multicopy plasmid formed on EMM2 plates at 26 °C for 5 days. Scale bar, 1 cm. Δ *ams2* cells carrying the integrated SpCENP-A-GFP gene at the *lys1* locus were cultured in EMM2 at 26 °C and were fixed by methanol. The centromeric GFP signals were observed under the condition that the indicated gene on the multicopy plasmid was overproduced. Representative sets of a cell at early G2 phase and a binucleated cell are shown in the right part: DAPI-staining (blue), GFP (green), and differential interference contrast (DIC). Scale bar, 10 μ m.

When the *Ams2*⁺ gene on a multicopy plasmid was added back into Δ *ams2* cells carrying the native promoter-driven SpCENP-A-GFP gene, the strength of the centromeric SpCENP-A-GFP signals had recovered to the level of that in wild-type cells as expected, whereas in the control cells carrying the empty vector, cell cycle-dependent SpCENP-A-GFP localization was observed. Dispersed GFP signals were observed in the G2 cells and centromerically localized GFP signals were seen in the binucleated cells (in the M, G1 or S phase). When histone H4 was overproduced, the strength of SpCENP-A-GFP signals at the centromeres had partly recovered, although the signals were still weaker than those in the cells carrying pAms2. In contrast, H3 overproduction reduced the amount of SpCENP-A localization, even in the binucleated Δ *ams2* cells.

Taken together, these findings suggest that *Ams2* ensures the replication-coupled incorporation of SpCENP-A, at least in part by promoting histone transcription. More importantly, in *Ams2*-deficient cells, SpCENP-A loading activity during the G2 phase was substantial, and this activity was found to be *Ams2*-independent (figure 6a(ii); Takayama & Takahashi, in preparation). Therefore, cells have at least two distinct cell cycle phases for SpCENP-A loading. In order to confirm that the loading point for SpCENP-A during the G2 phase actually exists in the presence of *Ams2*, we examined whether or not

Figure 8. The efficient loading of ectopically induced SpCENP-A onto the centromeres in G2-arrested cells. One additional copy of the SpCENP-A-GFP gene under the control of the thiamine-repressive *nmt1* promoter (Maudrell 1993) was integrated at the *lys1* locus of a *cdc25-22* ts mutant strain. Cells bearing the inducible SpCENP-A-GFP gene were pre-cultured in EMM2 at a permissive temperature (26 °C) in the promoter-repressed state (in the presence of 2 μ M thiamine). Cells were cultured at 26 °C for 15 h after the removal of thiamine and then cultured at a restrictive temperature (36 °C). Under this culture condition, newly induced SpCENP-A-GFP appeared after the cells had been arrested in the G2 phase. The upper panel shows the growth curve of the cells (diamonds) and the percentage of cells containing centromere-like GFP signals in the nucleus (circles). The lower panel shows representative merged cell images of GFP and DIC at 0, 2 and 5 h after the temperature shift. Scale bar, 10 μ m.

ectopically induced wild-type SpCENP-A protein could be loaded onto centromeres in G2-arrested cells (figure 8). To this end, we used the culture conditions under which the induced SpCENP-A-GFP protein appeared after the G2 arrest of the cells by *cdc25* mutation. At 2 h after the shift to 36 °C, cells stopped dividing, and were elongated at the G2 phase. At 3 h after the shift in temperature, SpCENP-A-GFP started to be expressed (data not shown) and to accumulate on a centromere-like dot in the nucleus. Thus, even after the passage of the S phase, the newly induced SpCENP-A protein was successfully loaded onto the centromeres.

We found that in wild-type cells, at least two loading phases for SpCENP-A are thought to exist during the cell cycle (figure 9a(i)). In *Ams2*-deficient cells, although SpCENP-A cannot be loaded at the S phase, a subsequent loading point at the G2 phase may act as a back-up pathway for the reloading of SpCENP-A protein before the cells enter the next M phase (figure 9a(ii)). However, the molecular basis for G2 loading remains to be determined. Although direct evidence has not yet been reported, we expected that the Mis6–Sim4 complex would be required for both the

S and G2 loading of SpCENP-A. Continuous inactivation of *mis6 ts* mutant gene from the G1 to G2 phase led to unequal chromosome segregation in the M phase (figure 9a(iii)), whereas Mis6 inactivation after the completion of the S phase did not produce missegregation in the subsequent M phase (figure 9a(iv); Saitoh *et al.* 1997). This phenotypic feature of the *mis6 ts* mutant is consistent with the postulated dual roles of Mis6 both in S and G2 loading. If this speculation is correct, then a *mis6 ts* mutant cell carrying *Ams2*-null mutation would be expected to show unequal chromosome segregation in the first M phase (figure 9a(v)). To test this possibility, we compared the timing of the appearance of asymmetrical nuclear division in a *mis6 ts* Δ ams2 double mutant with that in a *mis6 ts* single mutant. As shown in figure 9b, missegregation in the double-mutant cells, in which the loading pathway of the S phase was abolished by an *ams2*-null mutation, appeared significantly earlier than that in the single-mutant cells. This time lag of the appearance of missegregation was approximately 2–3 h, which is roughly equivalent to the duration of one cell cycle under these culture conditions. The present findings thus provide support for the idea that Mis6 is responsible for the G2 loading of SpCENP-A.

Figure 9. Two distinct cell cycle phases responsible for CENP-A loading in fission yeast. (a) The closed and open stars stand for the loading points of SpCENP-A in the S phase and the G2 phases, respectively. The striped zone represents the duration in which the cells were cultured at the restrictive temperature. The mitotic phenotype in the first M phase of each strain is schematically drawn in the right column. In the wild-type cells, at least two distinct loading opportunities for SpCENP-A were found to exist (i). In *Ams2*-null mutant cells, the replication-coupled loading of SpCENP-A in the S phase was perturbed (ii) (K. Takahashi *et al.* unpublished work). In *mis6 ts* mutant cells, both of the S and the G2 loading pathways were thought to be inactivated, because the continuous inactivation of the *mis6 ts* gene from the G1 to G2 phases is necessary for the appearance of the missegregation phenotype in the first M phase (iii). Inactivation of the *mis6 ts* gene after the passage of the S phase does not lead to chromosome missegregation in the subsequent M phase, probably because the S phase loading of SpCENP-A successfully takes place (iv). The above assumption predicts that the *mis6 ts* Δ ams2 double mutant cells would exhibit chromosome missegregation in the first M phase (v). (b) The percentage of asymmetric binucleated cells was monitored by DAPI staining after the temperature shift to 36 °C in *mis6-302 ts* mutant cells, under the condition that *Ams2* was expressed (circles: *mis6 ts* *ams2*-ON) or not expressed (squares: *mis6 ts* *ams2*-OFF). An *Ams2* shut off strain was constructed, in which a native promoter of the *ams2*⁺ gene was replaced with a repressible *nmr1* promoter (our unpublished material), and the shut off strain was then crossed with the *mis6-302 ts* mutant in order to create a conditional Δ ams2 *mis6-302* double mutant strain. The cells were pre-cultured in EMM2 at 25 °C (*Ams2* was expressed and *Mis6* was functional) and then the temperature was increased to 36 °C (circles: *Ams2* was expressed and *Mis6* was inactivated), or the cells were pre-cultured in EMM2 at 25 °C and then cultured in EMM2 in the presence of 2 μ M thiamine at 25 °C (*Ams2* expression was repressed and *Mis6* was functional) for 2 h before the temperature shift to 36 °C (squares: *Ams2* was repressed and *Mis6* was inactivated).

5. CONCLUDING REMARKS

Our current model of *Ams2* function is summarized in figure 10. We previously showed that *Ams2* is a putative member of GATA-type transcription factors that bind specifically to GATA-core DNA consensus sequences located in the promoter regions of the target genes (Chen *et al.* 2003a). We recently demonstrated that *Ams2* promotes histone gene activation at the S phase, probably as a transcriptional regulator (figure 10(i); Takayama & Takahashi, in preparation). *S. pombe* has three copies of histone H3 and H4 genes (*hht1*⁺, *hht2*⁺, *hht3*⁺, *hht4*⁺, *hht5*⁺ and *hht6*⁺), two copies of H2A genes (*hta1*⁺ and *hta2*⁺) and a single H2B gene (*htb1*⁺) in its genome (Matsumoto & Yanagida 1985). Although it remains to be determined whether or not *Ams2* binds directly to the promoter regions of histone genes, each upstream region of these histone genes, except for the *hht2*⁺ and *hht5*⁺ genes, contains DNA sequences that potentially match the GATA-core consensus sequence (HGATAR; H = T/A/C, R = A/G; Scazzocchio 2000). The *Ams2*-dependent supply of free histones at the S phase may be necessary for packing the SpCENP-A-containing centromere nucleosomes after DNA replication. Ectopic overproduction of histone H4, but not of H3, partly

Figure 10. A speculative model of Ams2 activity in SpCENP-A loading. Ams2 encodes a GATA-type transcriptional regulator that binds to the central core region of centromeres. Ams2 plays an essential role in the S-phase-specific transcriptional activation of core histone genes (i) (Takayama & Takahashi, in preparation). This activation leads to a transient increase in free histones in the cells, which could shift the chemical equilibrium to the formation of nucleosomes (black arrows), thus facilitating SpCENP-A incorporation. Ams2 is also likely to be involved in the selective incorporation of SpCENP-A-containing nucleosomes into the centromeres (broken arrows) (ii). This step may be achieved through the direct binding of Ams2 to the centromeres.

promoted SpCENP-A incorporation into the centromeres in the absence of Ams2 (figure 7). Therefore, lack of free histone H4 might account in part for the mislocalization of SpCENP-A in Ams2-deficient cells.

In addition to the transcriptional activation of histone genes, does Ams2 have other functions for facilitating SpCENP-A loading? As shown in figure 5*a*, histone H3 can be incorporated into the centromere nucleosomes instead of SpCENP-A in Ams2-null mutant cells. Thus, Ams2 probably maintains the centromere nucleosomes in a condition suitable for SpCENP-A localization, but not for histone H3 localization. This effect cannot be explained by a simple shift in the chemical equilibrium from free histones and SpCENP-A to nucleosomal formation because the transcriptional activation of the histone genes would also induce the formation of histone H3-containing nucleosomes as well as that of SpCENP-A-containing nucleosomes. Therefore, in addition to its suggested role as a transcriptional regulator (figure 10*i*), Ams2 is also involved in a selective incorporation of SpCENP-A (figure 10*ii*). Previously, we demonstrated that Ams2 is able to bind to the central core regions where SpCENP-A is specifically localized, and Ams2 appeared to function at the centromeres (Chen *et al.* 2003 *a,b*). This second step might be achieved through the direct binding of Ams2 onto the central core region, for example, by remodelling and/or modifying the centromere nucleosomes to maintain their suitability for SpCENP-A deposition. It

has been shown that GATA-type transcription factors exhibit nucleosome remodelling activity when they bind to the promoter regions of their target genes (Boyes *et al.* 1998; Cirillo *et al.* 2002; Chen *et al.* 2003*a*). Here, we propose that Ams2 assists in the efficient loading of SpCENP-A into centromere nucleosomes at the S phase by carrying out at least these two activities.

Although Ams2-deficient cells have been shown to exhibit a severe defect in the S phase loading of SpCENP-A, these cells can survive because they have an additional, back-up opportunity for SpCENP-A loading before entering the M phase. In this context, we would suggest that Mis6 is at least partly responsible for this back-up pathway based on the phenotypic features of *mis6* single- and *mis6 Δams2* double-mutant cells (figure 9). In this report, we discussed the flexibility of CENP-A localization and the plasticity of the centromere chromatin during the cell cycle in fission yeast. This chromatin plasticity might be governed by a common molecular mechanism in human neo centromere formation. When an authentic centromere happened to be deleted from a chromosome, a neo centromere could be established occasionally at a given time; namely, before entering anaphase in the subsequent M phase (figure 1*a*). This observation suggests that the assembly of the centromere proteins is able to take place within one round of the cell cycle in human cells, as demonstrated by the ability of the disrupted centromeric chromatin to reform rapidly before entering mitosis in *S. pombe*. The G2 loading activity of histone variants such as CENP-A may be evolutionarily conserved (Ahmad & Henikoff 2002; Tagami *et al.* 2004), and might at least in part account for the plasticity of centromere formation reported in higher eukaryotes.

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Discussion

T. Tanaka (*University of Dundee, Dundee, UK*). What happens to the expression level of CENP-A in Δ ams2 cells?

K. Takahashi. It was experimentally difficult to determine whether Ams2 affects a cell cycle-dependent CENP-A expression because the change of CENP-A mRNA level during the cell cycle was too subtle for detection by Northern analysis, especially in such a low synchronization sample of *ams2* deletant cells. However, using asynchronous culture samples, we observed the same expression level of CENP-A mRNA in Δ ams2 cells as that in wild-type cells. We have concluded so far that CENP-A expression is not severely impaired by Ams2 depletion.

J.-K. Heriche (*The Sanger Institute, Cambridge, UK*). What happens to CENP-A in the *mis6* mutant? What happens to the kinetochore?

K. Takahashi. The centromeric localization of CENP-A is significantly reduced at any stages of the cell cycle in the *mis6* mutant. The amount of CENP-A protein in the *mis6* mutant is equivalent to that in wild-type. Although the smear MNase pattern of the

kinetochore is completely abolished, other central core proteins such as Mis12 and SpCENP-C are localized quite normally on the Mis6-depleted kinetochores.

R. Allshire (*University of Edinburgh, Edinburgh, UK*). Are there S phase defects in Δ ams2 cells?

K. Takahashi. As far as we examined, surprisingly, there are no defects in the S phase in Δ ams2 cells. The FACS profile was comparable to that in wild-type cells. The genomic DNAs prepared from Δ ams2 cells were able to migrate into a gel in the pulse field gel electrophoresis. The Δ ams2 mutant was not sensitive to UV, and was also not sensitive to HU.

R. Allshire (*University of Edinburgh, Edinburgh, UK*). Is slow growth of Δ ams2 cells only due to centromere defects?

K. Takahashi. The major cytological defects of Δ ams2 cells were missegregation and hyper-condensation of mitotic chromosomes. The growth retardation of Δ ams2 cells was restored quite well by overproduction of histone H4 or CENP-A in the cells. Under these conditions, the centromeric localization of CENP-A was also recovered. Therefore, we believe that slow growth phenotype of Δ ams2 cells is mainly owing to the centromere defects caused by CENP-A mislocalization. We are currently trying to identify Ams2-target genes by DNA micro array analysis, and actually several candidate genes have been picked up, whose expressions are regulated by Ams2. Some of them may play essential roles in cell viability.