Biphasic Incorporation of Centromeric Histone CENP-A in Fission Yeast

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Abbreviations ChIP, Chromatin immunoprecipitation
Abstract

CENP-A is a centromere-specific histone H3 variant that is essential for kinetochore formation. Here we report that the fission yeast *Schizosaccharomyces pombe* has at least two distinct CENP-A deposition phases across the cell cycle: S and G2. The S phase-deposition requires Ams2 GATA factor, which promotes histone gene activation. In Δams2, CENP-A fails to retain during S, but it reaccumulates onto centromeres via the G2-deposition pathway, which is down-regulated by Hip1, a homolog of HIRA histone chaperon. Reducing the length of G2 in Δams2 results in failure of CENP-A accumulation, leading to chromosome missegregation. N-terminal GFP-tagging reduces the centromeric association of CENP-A, causing cell death in Δams2 but not in wild-type cells, suggesting that the N-terminal tail of CENP-A may play a pivotal role in the formation of centromeric nucleosomes at G2. These observations imply that CENP-A is normally localized to centromeres in S phase in an Ams2-dependent fashion and that the G2 pathway may salvage CENP-A assembly to promote genome stability. The flexibility of CENP-A incorporation during the cell cycle may account for the plasticity of kinetochore formation when the authentic centromere is damaged.
Introduction

The kinetochore is a multiprotein-DNA complex that is indispensable for chromosome segregation, and which normally forms on a single chromosomal locus, the centromere (Cleveland et al., 2003). Lack of a kinetochore or formation of multiple kinetochores on a chromosome may have deleterious effects on mitosis (Karpen and Allshire, 1997; Amor and Choo, 2002; Henikoff and Dalal, 2005). CENP-A represents the most likely candidate for the epigenetic ‘mark’ responsible for the maintenance of centromere identity (Black et al., 2004; Black et al., 2007). As reformation of CENP-A-containing nucleosomes after DNA synthesis is thought to be a prerequisite for mitotic kinetochore assembly, precise targeting of CENP-A into a single, restricted locus on each chromosome prior to cell division is essential for cell viability (Takahashi et al., 2005). At least three components that affect CENP-A localization, the Mis16-Mis18 complex (Hayashi et al., 2004; Fujita et al., 2007), Mis6-Sim4 complex (Takahashi et al., 2000; Pidoux et al., 2003), and Ams2 GATA-type transcription factor (Chen et al., 2003a), have been identified in the fission yeast Schizosaccharomyces pombe, which is an ideal model organism in which to study complex centromere structure and function (Takahashi et al., 1992; Karpen and Allshire, 1997).

Which phase of the cell cycle is utilized for CENP-A incorporation remains controversial. During S phase, canonical core histones have been suggested to be deposited into duplicated DNAs in a semi-conservative fashion (Tagami et al., 2004; Natsume et al., 2007). Experiments using fluorescence recovery after photobleaching (FRAP) demonstrated that CENP-A of the
budding yeast *Saccharomyces cerevisiae* is recruited to centromeres coincident with DNA synthesis (Pearson *et al.*, 2004), presumably reflecting disassembly and reassembly of centromeric nucleosomes at the replication fork. In contrast, studies performed in human cells (Shelby *et al.*, 2000; Jansen *et al.*, 2007) and in *Drosophila melanogaster* (Ahmad and Henikoff, 2001; Sullivan and Karpen, 2001; Schuh *et al.*, 2007) indicated that CENP-A is incorporated in a replication-independent manner, although the molecular components and physiological significance of this pathway remain elusive. Here, we report that Δams2 cells are defective in the retention of Cnp1, *S. pombe* CENP-A, at S phase, but are able to survive through Cnp1 incorporation using the replication-independent pathway at G2. Our observations indicated that the G2-deposition of Cnp1 is mechanically distinct from the S-deposition and could act as a salvage pathway that enables unincorporated Cnp1 to reassociate to the centromeres before cells go through subsequent lethal mitosis.
Materials and Methods

General techniques, strains, antibodies and plasmids
The techniques and media used for manipulation of fission yeast were described previously (Saitoh et al., 1997; Chen et al., 2003a). The genotypes of strains and procedures used for gene disruption are described in Table S1. Anti-Cnp1 polyclonal antibody was generated by immunizing rabbits with GST-tagged Cnp1. The minichromosome loss assay was performed as described previously (Takahashi et al., 1994). To construct pRep41 based-plasmids carrying the N-terminally deleted derivative of the C-terminal tagged Cnp1-GFP gene, the ORF of the deleted derivatives of Cnp1 gene was amplified by PCR and inserted into the SalI-NotI cloning sites of pGP4110 in frame. pGP4110 (a derivative of pGP110 (Saitoh et al., 2005)) is a multicopy plasmid for the C-terminal GFP tagging under the control of the nmt41 promoter. PCR primer sequences used for the PCR amplification were as follows:

Full length, 5’GTCGACATGGCAAAGAAATC3’/5’GCGGCCGCTAGCACCACGAATCC3’;

D5, 5’GTCGACATGGCTGAGCCTGG3’/5’GCGGCCGCTAGCACCACGAATCC3’;

D10, 5’GTCGACATGGATCCTATTCCAC3’/5’GCGGCCGCTAGCACCACGAATCC3’;

D15, 5’GTCGACATGCCACGTAAAAAG3’/5’GCGGCCGCTAGCACCACGAATCC3’;

D20, 5’GTCGACATGTATCGTCCAGGTAC3’/5’GCGGCCGCTAGCACCACGAATCC3’.

Construction of GFP-Cnp1 strains
To construct a pBluescript KS-based plasmid carrying the N-terminal tagged GFP-Cnp1 gene (PS1), HindIII-BamHI cloning sites were created behind the first
Met of the ORF of the Cnp1 gene. The ORF of the EGFP gene (Clontech Laboratories, Heidelberg, Germany) amplified by PCR using primers containing HindIII and BamHI sites was inserted into the cloning sites in frame. A cnp1-1\textsuperscript{ts} strain (SP1786, ura4 Δcnp1::ura4\textsuperscript{+} lys1\textsuperscript{+}::cnp1-1) was transformed with PS1 and the transformants were grown at 22°C (permissive temperature for cnp1-1) on EMM2 plates in the presence of 1mg/ml 5-fluoroorotic acid, permitting identification of colonies where recombination between the GFP-Cnp1 gene and the disrupted Cnp1 gene at the authentic locus has led to loss of the Ura4 marker and return to uracil auxotrophy. All transformants were viable at 36°C. Replacement of the GFP-Cnp1 gene with the authentic gene in the transformants was confirmed by genomic Southern analysis. The transformants were then crossed with wild-type strain (SP171) to remove the cnp1-1 gene at the lys1 locus, and the resultant GFP-Cnp1 strain (SP1769) was used for the experiments shown in Figure 3 and 4.

To generate the lys1\textsuperscript{+}::GFP-Cnp1 strain (SP1468), a 2.4kb KpnI-EcoRI fragment containing the GFP-Cnp1 gene was subcloned into the plasmid pTK2 designed for integration of the inserted DNAs into the lys1\textsuperscript{+} locus. A lys1\textsuperscript{−} strain (SP91) was transformed with the resultant integration plasmid (PS2) and plated on EMM2 lacking lysine to select the integrants. The additional integration of the GFP-Cnp1 gene at the lys1\textsuperscript{−} locus in the transformants was confirmed by genomic Southern analysis.

**Microscopy**

For DAPI staining in Figure 5, cells were cultured in EMM2 containing the appropriate supplements with or without 2 μM thiamine at 33°C. Cells were
fixed in methanol at -80°C, washed with PBS, and mixed with 200 ng/ml DAPI. Images were collected using VB-6000/6010 (Keyence, Osaka, Japan) with a 100x 1.45 numerical aperture alpha Plan-FLUAR objective (Carl Zeiss, Jena, Germany). For Live cell analyses, cells were cultured in EMM2 containing the appropriate supplements and then embedded in 1.5% low melting point agarose in EMM2 with supplements on glass-bottomed dishes. Images of living cells were obtained using a Leica ASMDW live cell imaging system microscope with a 100x 1.40 numerical aperture ACX PL Apo objective (Leica Microsystems, Wetzlar, Germany). Images collected every 0.3 μm along the Z-axis were processed with the non-blind three-dimensional deconvolution algorithm using ASMDW software. The projected images were converted to kymographs with Metamorph software (Universal Imaging, Downingtown, PA, USA). The fluorescent intensity of the centromeric GFP-dots and that of the nuclear GFP background were calculated by Image Quant IL (GE Healthcare, Buckinghamshire, UK) after the background (signals outside of the cell) titration. For the experiments shown in Figure 2, a total of 513 videos was recorded and analysed individually. Cells were classified into five cell cycle stages: stage I, unseptated binucleate cells (M/G1); stage II, septated binucleate cells (G1/S); stage III–V, single nuclear cells <7.1 μm (III, S/early G2), 7.1–10.5 μm (IV, mid G2) or >10.5 μm (V, late G2) in length. As the average septated cell length of Δhip1 was 36% longer than that of wild-type controls, we used the following values as the criteria of G2 classification for Δhip1: stage III–V, single nuclear cells <9.6 μm (III), 9.6–14.3 μm (IV) or >14.3 μm (V) in length.
Chromatin immunoprecipitation (ChIP)

For the experiments shown in Figure 1C, cells in the following genetic backgrounds (lys1‘::Cnp1-GFP represents the native promoter-driven Cnp1-GFP gene additionally integrated at the lys1 locus) were cultured in YES at 26°C and then shifted to 36°C for 3.5 h (cdc25-22 lys1‘::Cnp1-GFP; SP1234, cdc25-22; YTP379, cdc25-22 Δams2 lys1‘::Cnp1-GFP; SP1751 and cdc25-22 Δams2; YTP355 for late G2), 4.5 h (cdc10-129 lys1‘::Cnp1-GFP; SP1628, cdc10-129; SP2959 for G1, and cdc22-C11 lys1‘::Cnp1-GFP; SP1633, cdc22-C11; SP2962 for S) or 5.5 h (cdc10-129 Δams2 lys1‘::Cnp1-GFP; SP1699, cdc10-129 Δams2; SP2960 for G1, and cdc22-C11 Δams2 lys1‘::Cnp1-GFP; SP1704, cdc22-C11 Δams2; SP2963 for S). Wild-type (SP91), lys1‘::Cnp1-GFP (SP92), Δams2 (YTP155) and Δams2 lys1‘::Cnp1-GFP (SP75) cells were cultured in YES at 33°C for asynchronous samples, and then the early G2 cells were prepared by centrifugal elutriation (Avanti HP-20XP, JE-5.0 elutriation rotor; Beckman Coulter, Fullerton, CA, USA). ChIP assays were performed using anti-GFP monoclonal antibody (Roche, Indianapolis, IN, USA) and Dynabeads anti-Mouse IgG (Dynal Biotech, Oslo, Norway), or anti-Cnp1 polyclonal antibody and Dynabeads anti-Rabbit IgG (Dynal Biotech) as described previously (Takayama and Takahashi, 2007). The DNA samples prepared from 3% formaldehyde-fixed chromatin solutions or immunoprecipitated fractions were analysed by real-time PCR using an ABI 7000 Sequence Detection System and Power SYBR Green PCR master mix (Applied Biosystems, Foster City, CA, USA). The ratios of ChIP signals for cnt2 in the Δams2 background were quantified as intensities relative to those in the Δams2‘ background. In all samples, intensities of ChIP signals for otr (Saitoh et al., 1997) and act1 probes (background
controls) were less than 3.6 and 0.2% of those for the cnt2 probe, respectively. PCR primer sequences were as follows:

cnt2, 5’AAAGCAACACGACGTAAACCTTGTAA3’/5’TGCCTCTTATATGCGGCTTA3’;
imr1, 5’CCTTACTGAAAAATTTGTGG3’/5’GCTGAGGCTAAGTATCTGTT3’;
otr (dg), 5’CATGGAACTACGTCAGAGGTGG3’/5’TGCCCTGTTCACCTATCTAATTGG3’;
act1, 5’CTTTCTACACGAGCTTCGTGGG3’/5’GAGTCCTCTTCACCGTGGAT3’.
Results

Cell cycle-dependent centromeric localization of Cnp1 in Δams2

Ams2 was originally identified as one of the multicopy suppressors in Cnp1 ts mutation (cnp1-1) and was shown to be required for the localization of Cnp1 at centromeres (Chen et al., 2003a). Recently, we demonstrated that Ams2 promotes S phase-specific activation of core histone genes (Takayama and Takahashi, 2007). Periodic histone transcription may be important for appropriate Cnp1 incorporation. Surprisingly, Ams2 was shown to be dispensable for cell viability, although its depletion causes severe mislocalization of Cnp1. To clarify the behavior of Cnp1, we performed live cell analyses using wild-type or Δams2 carrying the C-terminal GFP-tagged Cnp1 gene driven by the native promoter (Figure 1A and B, Supplemental material Figure S1, Videos 1-4). In Δams2, we found that Cnp1-GFP is located on centromeres in a cell cycle-dependent fashion. Cnp1-GFP began to accumulate onto centromeres from the latter half of G2, and signal intensities peaked around late G2 and M phases. The GFP signals then disappeared after cell division. Under these conditions, S phase occurs during septum formation and cell division in both wild-type (Rustici et al., 2004) and Δams2 cells (Supplemental material Figure S2). Thus, Ams2 is required for appropriate centromeric localization of Cnp1-GFP from late S phase to mid G2 phase.

Figure 1B shows the data quantifying the changes in fluorescence intensity of Cnp1-GFP throughout the cell cycle in the mixture of wild-type and Δams2 in the same frame. In the image of both of the wild-type and the Δams2 cell,
the centromere fluorescence of Cnp1 signals appeared to rapidly drop as chromosomes segregate (Figure 1B and Supplemental material Figure S1, asterisks) but seemed to increase in fluorescence shortly thereafter. We found that the intensities decreased roughly half at the onset of M phase, which may reflect the instability of Cnp1 at (Mellone and Allshire, 2003) and/or declustering of the mitotic centromeres (Nabeshima et al., 1998). We also found that, in the Δams2 cell, a short uptake of Cnp1 incorporation occurred at G1/S phase (Figure 1B and Supplemental material Figure S1B, arrows). Because Cnp1 is transcribed at G1/S phase prior to core histone transcription (Takahashi et al., 2000) in the Ams2-independent manner (Takayama and Takahashi, 2007), the uptake may correspond to G1/S deposition of Cnp1. Full boost of Cnp1 may require the following Ams2-dependent activation of the core histone expression (Takayama and Takahashi, 2007). The signal intensity reached to the maximum from mid G2 to late G2 phase in wild-type cell, whereas it reached just before M phase in Δams2 cells.

We performed chromatin immunoprecipitation (ChIP) analysis to confirm that the dynamics of GFP actually reflect the association of Cnp1-GFP with the DNA of the centromere (Figure 1C). The amount of centromere-bound Cnp1-GFP at late G2 in the Δams2 background was almost equivalent to that in wild-type controls, whereas those at G1, early S and early G2 phases were reduced to 49, 27 and 5%, respectively. In the Δams2 background, Cnp1-GFP was associated correctly with the central centromere region (cnt1). These observations indicate that, in Δams2, the centromeric Cnp1-GFP is diminished during S phase, and then disappears. Inhibition of DNA synthesis by addition of hydroxyurea
(HU) blocked the disappearance of Cnp1-GFP signals in Δams2 (Supplemental material Figure S3), indicating that the dissociation of Cnp1-GFP in Δams2 requires completion of DNA replication. By ChIP analysis using anti-Cnp1 polyclonal antibody, we next examined the cell cycle change in the centromeric association of non-tagged Cnp1 that is expressed from the authentic locus. The total amount of centromere-bound Cnp1 in Δams2 relative to that in wild-type controls was reduced to 49% in early S and 46% in early G2 phase, while there were smaller changes in late G2 (71%) and G1 (80%) phases (Figure 1C). Therefore, Ams2 is required to prevent the reduction of Cnp1 from S phase to early G2 phase. Considering the inconsistency with the drastic change of the centromeric Cnp1-GFP, C-terminal GFP-tagging may reduce the affinity of Cnp1 with the centromeric DNA to some extent, and thus may somewhat exaggerate the impacts on Cnp1 localization by deleting Ams2.

Two distinct phases of Cnp1-GFP incorporation during the cell cycle: S and late G2

The dynamic behavior of Cnp1-GFP in Δams2 cells highlights substantial Ams2-independent Cnp1 localization activity during the later half of G2 (Figure 1 and Supplemental material Figure S1B). There are likely to be at least two distinct cell cycle phases in which centromeric Cnp1 localization in fission yeast. To confirm that Cnp1 incorporation during G2 phase actually occurs in the presence of Ams2, we performed Cnp1 reloading assay using C-terminal GFP-tagged Cnp1ts protein (Chen et al., 2003a). The Cnp1ts-GFP gene driven by the native promoter was integrated into the genome of wild-type or Δams2 cells,
and the resultant cells carried both the Cnp1<sup>ts</sup> gene and the authentic gene, but only the ts protein was visible by GFP-tagging. The Cnp1<sup>ts</sup> protein with a mutation (L87Q) in the middle of the α2 helix, corresponding to the CENP-A targeting domain in humans (Black et al., 2004; Black et al., 2007), showed a temperature-dependent localization defect (Chen et al., 2003a). To examine the timing of Cnp1-GFP incorporation, we followed Cnp1<sup>ts</sup>-GFP dynamics after shifting from 36°C to a low temperature of 22°C. For comparison between wild-type and Δams2, the cells were classified into five morphological categories (stage I to V; see Materials and methods) based on the number of nuclei, the appearance of the septum and cell length. Under the condition used, the cell cycle distribution of Δams2 cell culture was roughly comparable with that of wild-type controls (Figure 2C, upper). Mass analyses of Cnp1 reloading assay indicated that Cnp1<sup>ts</sup>-GFP protein was incorporated around S phase in 75.4% of cases in wild-type cells (181 of 240 cells examined; Figure 2, A and C, lower: stages II–III, Video 5). In the remaining 24.6% of the wild-type cells, Cnp1<sup>ts</sup>-GFP accumulated at late G2 (Figure 2, B and C, lower: stage V, Video 6). These observations indicated that G2-loading of Cnp1-GFP is not an exceptional phenomenon occurring only in Ams2-deficient cells. Therefore, wild-type cells exhibit at least two distinct peaks of Cnp1<sup>ts</sup>-GFP incorporation across the cell cycle, at S and late G2 phases, with no Cnp1<sup>ts</sup>-GFP incorporation in M/G1 (Figure 2C, lower: stage I) or mid G2 (stage IV).

In contrast, in Δams2, reloading of Cnp1<sup>ts</sup>-GFP around S phase was reduced to 17.7% (31 of 175 cells examined; Figure 2C, lower: stages II–III), whereas that during late G2 increased to 82.3% (stage V). In most cases in Δams2,
Cnp1-GFP appeared as dispersed signals in the nucleus but failed to accumulate into centromeres at S phase, which was then reloaded at late G2 (Video 7). Thus, Ams2 is critical for S phase-dependent but not -independent deposition of Cnp1-GFP onto centromeres. As shown in Figure 1C, the amount of authentic Cnp1 on centromeres in Δams2 cells was reduced to approximately half of that in wild-type cells at S and early G2. This reduction may be a consequence of the failure in the centromere loading of newly synthesized Cnp1 proteins in Δams2 cells in which pre-existing Cnp1 is retained on centromeres.

**Hip1-dependent repression of Cnp1 deposition during G2 phase**

The above observation raises questions regarding the components of the G2-deposition pathway. We examined whether the fission yeast homolog of HIRA, Hip1 (Blackwell *et al.*, 2004), is involved in Cnp1 deposition outside S phase; *Xenopus* HIRA is critical for the nucleosome assembly pathway independent of DNA synthesis (Ray-Gallet *et al.*, 2002), and human HIRA was identified as a chaperone for H3.3-nucleosome (Tagami *et al.*, 2004), which is deposited in a replication-independent manner (Ahmad and Henikoff, 2002). We generated a Hip1-null strain, which was viable as reported previously (Blackwell *et al.*, 2004). Although the doubling time of Δhip1 cells (4.4 hr) was 1.5 times longer than that of wild-type cells (3.0 hr) in YES at 26°C, the cell cycle progression of Δhip1 cells seems comparable with that of wild-type using the five morphological categories; judging from the timing of the appearance of Mrc1 protein, the S phase in Δhip1 cells appeared to occur during the septum formation as same as in wild-type and Δams2 (*data not shown*). This shows that the G2 phase
starts with the normal timing in Δhip1 cells and the cell length distribution and
the septum formation can be used as good hallmarks for the cell cycle
progression in the absence of Hip1. Native promoter-driven Cnp1-GFP protein
was localized at centromeres throughout the cell cycle in the Δhip1 background
(data not shown). However, we found that Cnp1\textsuperscript{ts} reloading occurred throughout
G2 in cells lacking Hip1 (Figure 2C). In addition to Cnp1\textsuperscript{ts}-GFP incorporation in
late G2 (stage V), that in the first half of G2 (stages III-IV) occurred. Thus, in
wild-type cells, the replication-independent, G2-deposition activity of Cnp1-GFP
during the normal cell cycle is likely under active repression and could be
controlled by Hip1, an S. pombe homolog of HIRA protein. Alternatively, Hip1
may promote the refolding and/or reassembly of Cnp1\textsuperscript{ts} protein to facilitate the
reloading. It is also possible that the lack of Hip1 activity could just create
additional loading sites for Cnp1 as a secondary consequence, not an active
mechanism to exclude Cnp1. The additional deletion of Hip1 in Δams2 cells did
not suppress the growth retardation but rather showed the synthetic growth
defect (data not shown), suggesting that Hip1 and Ams2 have the additional roles
in cell growth besides regulating Cnp1 loading.

**N-terminal GFP-tagged Cnp1 is functional in wild-type but not in Δams2**

As replacement of the authentic Cnp1 gene with the C-terminal tagged
Cnp1-GFP gene causes cell growth retardation (data not shown), we generated
wild-type cells expressing N-terminal tagged GFP-Cnp1 at the authentic locus
and tested that it is fully functional. This GFP-Cnp1 integrant grew normally at
any temperatures tested and its cell viability was comparable with that of
wild-type controls (Figure 3A and *data not shown*). The sensitivity of the GFP-Cnp1 strain to the spindle poison, carbendazim (CBZ), was the same as that of the wild-type control (Figure 3B). Fluorescence microscopy indicated co-localization of the signal of GFP-Cnp1 with that of the centromere-marker, SpMif2-CFP, throughout the cell cycle (*data not shown*). The results of ChIP analysis using anti-GFP antibody indicated that GFP-Cnp1 binds to the central core DNAs (*cnt* and *imr*) but not the outer repeats (*otr*) of the centromere as does authentic Cnp1 (Figure 3C), suggesting that the N-terminal GFP-tag does not prevent the localization of Cnp1 to the centromere. However, the mitotic loss rate of a linear minichromosome, CN2, in the GFP-Cnp1 integrant cells is threefold greater than that in wild-type cells (Figure 3D). The total expression level of Cnp1-GFP protein additionally integrated at *lys1* locus was severalfold greater than that of endogenous Cnp1, while that of GFP-Cnp1 expressed from the *lys1* locus or from the native locus was comparable to that of authentic Cnp1 protein (Figure 3E). The level of expression of each tagged Cnp1 protein in the Δ*ams2* background was comparable to that in wild-type cells (Figure 3E).

The above results indicated that the GFP-Cnp1 gene replacing the authentic Cnp1 gene was functional in wild-type cells; at least the native chromosomes can be maintained without detectable defects on mitosis. Surprisingly, the GFP-Cnp1 integrant became synthetic lethal with the Δ*ams2* strain. Tetrad analysis indicated that spores of Δ*ams2* expressing GFP-Cnp1 formed micro-colonies but soon ceased growing. Microscopic observation of the micro-colonies revealed that GFP-Cnp1 signals failed to accumulate on centromere-like dots in Δ*ams2*, and that frequent chromosome missegregation
occurred (Figure 4A). The intact N-terminal tail may be important for association of Cnp1 with the centromere in Ams2-deficient cells. Alternatively, it is formally possible that the cell viability is apt to be influenced by a small decrease in GFP-Cnp1 protein in the Δams2 background. Additional integration of the GFP-Cnp1 gene at the lys1 locus in Δams2 cells resulted in recovery of cell viability to the level in Δams2 cells expressing non-tagged Cnp1 (data not shown). However, the centromeric localization activity of GFP-Cnp1 was clearly defective (Figure 4A). Therefore, the growth arrest observed in Δams2 spores expressing GFP-Cnp1 is not due to a dominant negative effect of GFP-Cnp1 on Ams2-deletion but is presumably due to the failure of GFP-Cnp1 retention at centromeres in Δams2 cells. One interesting possibility suggested by the results shown in Figure 3 and Figure 4A is that the N-terminal tagging specifically inhibits G2-deposition but not S-deposition of Cnp1. To test this possibility, we next attempted to generate wild-type and Δams2 cells expressing N-terminal tagged GFP-Cnp1ts protein expressed from the lys1 locus for reloading assay. However, as the centromeric localization activity of GFP-Cnp1ts protein co-expressed with authentic Cnp1 was too low for quantitative analysis in the reloading assay, even in the wild-type background (data not shown), it was not possible to examine the above possibility in this study.

As another approach to clarify the importance of the N-terminal tail for the Ams2-independent centromeric retention of Cnp1, we examined the subcellular localization of the N-termially deleted derivatives of Cnp1-GFP (Figure 4B). Overexpressed full-length Cnp1-GFP and D5 derivative accumulated on the centromeres in both wild-type and Δams2 cells, while D15
derivative exhibited the nuclear localization. Because D20 derivative lost the nuclear localization activity, the five amino acids sequences (PRKKR) may contain the nuclear localization signal (NLS), in which the basic cluster RKKR is an apparent candidate matching to the consensus sequence of NLS (Jans et al., 2000). Interestingly, while D10 derivatives were imported into the nucleus both in wild-type cells and Δams2 cells, they were located on the centromeres in wild-type cells but not well in Δams2 cells, indicative of the function of the N-terminal tail of Cnp1 for Ams2-independent centromeric localization.

**G2-deposition pathway ensures stable chromosome transmission in cell division when S-deposition is impaired**

The above results indicated that wild-type cells exhibit biphasic incorporation of histone variant Cnp1 during the cell cycle, which could be regulated differently. The next question is why *S. pombe* cell has evolved two distinct deposition pathways. Δams2 cells are viable, although Cnp1 could not retain efficiently at the centromere during S phase. Therefore, the subsequent incorporation during G2 phase could function as another opportunity for replenishing the duplicated centromere DNA with newly synthesized Cnp1 before cell division. If this hypothesis is correct, shortening of G2 phase by introduction of *wee1* mutation (Hayles and Nurse, 1992) should reduce cell viability in the absence of Ams2. To test this possibility, we generated *wee1-50* cells carrying the *ams2+* gene under the control of a repressible promoter. When Ams2 was expressed, the cells showed the *wee* phenotype, but grew normally with equal chromosome segregation in M phase (Figure 5A, *ams2-ON*). On the other hand, the cells exhibited a high
frequency of unequal chromosome segregation with the \textit{wee} phenotype (Figure 5A, \textit{ams2-OFF}) and did not survive when Ams2 was repressed (Figure 5B). The centromeric localization of Cnp1-GFP was markedly reduced in ams2-deficient \textit{wee1-50} mutant in comparison with \textit{wee1-50}, ams2-deficient or wild-type controls (Figure 5C). In contrast, \textit{\Delta ams2 cdc25-22} double mutant with prolonged G2 phase (Hayles and Nurse, 1992) showed much better growth than the single \textit{\Delta ams2} mutant (Figure 5D). These observations indicated that the viability of Ams2-deficient cells is influenced by the length of the G2 phase. Therefore, when S-deposition is impaired, G2 phase could function as the recovery period for Cnp1 deposition, and this safety mechanism based on the biphasic incorporation could ensure high fidelity of chromosome transmission in mitosis.
Discussion (1,615 words)

Relationship between Cnp1 deposition and histone expression

There is a great deal of evidence that perturbation of histone transcription impairs centromere functions, resulting in chromosome missegregation (Meeks-Wagner and Hartwell, 1986; Holmes and Mitchell Smith, 2001; Maruyama et al., 2007). The results of the present study indicated that Ams2 GATA factor prompting core histone genes activation at S phase (Takayama and Takahashi, 2007) ensures simultaneous Cnp1 deposition onto centromeres in fission yeast. While depletion of Ams2 results in reduction of the binding of Cnp1 to centromeres and chromosome missegregation, increasing its dosage restores the association of Cnp1 mutant protein (Cnp1<sup>ts</sup>) with centromeres (Chen et al., 2003a). Supply of histone H4, a binding partner of Cnp1, by transformation with a multicopy plasmid partially restored the centromeric localization of Cnp1 in Δams2 cells, whereas overproduction of histone H3, which competes with Cnp1 for H4 binding, is toxic to Δams2 cell growth (Chen et al., 2003a; Takahashi et al., 2005). These observations indicated that appropriate regulation of histone transcription at S phase, especially up-regulation of H4 transcription, is required for efficient incorporation of Cnp1 onto centromeres. As the supply of histone H4 in Δams2 cell does not fully suppress the defect of Cnp1-GFP localization (Takahashi et al., 2005), in addition to up-regulation of histone transcription, Ams2 may have other roles in stabilizing the centromeric retention of Cnp1. For example, binding of Ams2 to the centromere may remodel the centromeric nucleosomes, which is a prerequisite for suitable incorporation of Cnp1(Chen et
and/or other as yet unidentified transcriptional targets of Ams2 may be important for the formation/retention of Cnp1-containing nucleosomes at S phase.

Because the intracellular levels of histone proteins are not markedly affected in Δams2 (Takayama and Takahashi, 2007), significant reduction of histone gene transcription at S phase may alter the relative ratio of free histones carrying the modification pattern suitable for Cnp1 formation/retention at S phase (Hayashi et al., 2004; Fujita et al., 2007). Recently, we showed that the level of *S. pombe* histone mRNAs is likely to be controlled as a combination of Ams2-dependent transcriptional activation at S phase and Ams2-independent constitutive transcription throughout the cell cycle (Takayama and Takahashi, 2007). Intriguingly, this basal histone transcription is normally down-regulated by Hip1 (Blackwell et al., 2004; Takayama and Takahashi, 2007), which we showed silences the G2-deposition pathway for Cnp1 in this study. As the net retention of Cnp1 at the centromere would be regulated by a dynamic equilibrium between incorporation and releasing of Cnp1, it is possible that Ams2 and Hip1 are playing roles in the equilibrium but not as loading factors per se.

*Dynamics of Cnp1 retention at the centromere*

The incorporation of newly synthesized and/or misloaded CENP-A onto the centromere and releasing of pre-existing CENP-A would affect the centromeric localization. Therefore, the steady state distribution of Cnp1 we observed in this study should be dissected into incorporation processes (formation/stabilization
of Cnp1-nucleosomes and/or accessing/loading of Cnp1 to the centromere) and releasing processes (disassembly/destabilization of Cnp1-nucleosomes and/or degradation of Cnp1 at the centromere) in the future study. Here we demonstrated that there are at least two separate phases of Cnp1 deposition onto the centromere across the cell cycle in S. pombe: one during DNA synthesis, where newly synthesized DNA is re-chromatinized following the passage of the replication fork, and a second phase that likely occurs during late G2, just before chromosome segregation in mitosis. Importantly, both deposition pathways may be mechanically distinct because N-terminal GFP-tagging impairs the centromeric retention of Cnp1 in Δams2 cells but not in wild-type cells. One plausible explanation is that N-terminal tagging inhibits Cnp1 loading onto the centromere at G2 but not S phase. Alternatively, GFP-tagging may not inhibit Cnp1 deposition itself, but may prevent formation/stabilization of the functional centromeric nucleosomes after G2-deposition. It is also formally possible that C-terminal GFP tagging specifically inhibits Cnp1 deposition from late M to G1 phase, at which stage newly synthesized GFP-CENP-A has been shown to be loaded in human cells (Jansen et al., 2007). Our results suggest that GFP tagging may mask some of the functional CENP-A incorporation/releasing pathways even though the GFP-tagged protein can be replaced successfully with the authentic gene product in the wild-type background.

In addition to G2-deposition, the quantification of the centromeric Cnp1-GFP in Δams2 cells revealed that a short uptake of Cnp1 deposition in G1 and/or early S phase exists (Figure 1B and Supplemental material Figure S1B, arrows). This may correspond to G1-deposition of CENP-A documented in
human tissue culture cells (Jansen et al., 2007). Although we could not detect G1/early S-deposition of Cnp1\(^{ts}\) protein in our reloading assay (Figure 2), the behavior of the Cnp1\(^{ts}\)-GFP carrying a specific mutation (L87Q) might not be accurately reflecting that of endogenous wild-type Cnp1. Because Cnp1 signals in \(\Delta\)ams2 cells seemed to disappear shortly after the incorporation in G1/S phase, there may be the active destabilization of Cnp1 in S phase; in the budding yeast, the degradation of Cse4 protein was shown to be an important determinant for the centromeric localization (Collins et al., 2004).

*The evolutionary conservation of CENP-A dynamics*

The cell cycle timing of CENP-A loading appears not to be conserved evolutionarily. To date, the following have been proposed: G1- (Jansen et al., 2007) or G2-deposition (Shelby et al., 2000) in human cells, G2-deposition (Ahmad and Henikoff, 2001; Sullivan and Karpen, 2001) in Drosophila cells in culture, anaphase-deposition in Drosophila syncytial nuclear division (Schuh et al., 2007), and S-deposition in budding yeast (Pearson et al., 2004) and the primitive red alga (Maruyama et al., 2007). Pioneering work using human cells (Shelby et al., 2000) indicated that the incorporation of CENP-A occurs primarily during G2 phase. However, recent pulse-chase experiments of CENP-A protein using SNAP tag technology clearly demonstrated that, in human cells, loading of newly synthesized CENP-A occurs in a discrete cell cycle window in early G1 (Jansen et al., 2007). hMis18\(\alpha\), hMis18\(\beta\) and M18BP1/hsKNL2 proteins, essential components of CENP-A loading pathways, display a pattern of centromeric localization coincident with CENP-A assembly in G1 (Fujita et al., 2007; Maddox
et al., 2007). It is noteworthy that the pattern of fission yeast Mis18-GFP was reported to be diffused just before mitosis and restored as the centromere-like dots in late anaphase (Fujita et al., 2007), indicating that the fission yeast Mis18 is located at centromeres throughout most of the cell cycle, including telophase, G1, S and almost all G2 phase. This differs from the localization pattern of human Mis18 proteins, which are associated with centromeres for only a short period in the cell cycle coincident with the timing of CENP-A deposition (Jansen et al., 2007). The prolonged centromeric localization of the Mis18-complex in fission yeast may enable Cnp1 to be loaded to centromeres throughout most of the cell cycle.

The stable maintenance of pre-existing CENP-A after passage of S phase in human cells (Jansen et al., 2007) and in Drosophila cells (Sullivan and Karpen, 2001) are also in sharp contrast with the behavior of Cse4, the budding yeast CENP-A (Pearson et al., 2004); the properties of the centromeric localization of Cse4 are rather dynamic, with most pre-existing Cse4 at centromeres replaced with non-centromeric Cse4 during S phase (Bloom, 2007). In the early Drosophila blastoderm embryo, in less than 2 min after photobleaching, more than 100% of the initial fluorescent intensity of GFP-tagged CID, the Drosophila homolog of CENP-A, was shown to be recovered into centromeres in anaphase (Schuh et al., 2007). The observation suggests the dynamic behavior of centromeric histone in some situations; a complete exchange of pre-existing CID takes place in a very short period during anaphase. Even in the same species, CENP-A may behave differently according to developmental stages or specific environmental conditions.
Physiological significance of biphasic incorporation of Cnp1

As the wee1 mutant with a shortened G2 phase showed no apparent defects in Cnp1 localization (Figure 5C), S-deposition appears to be the primary pathway for loading in S. pombe, and G2-deposition has likely evolved as a secondary pathway to increase fidelity in this organism. Interestingly, GFP-tagging or a partial deletion of the N-terminal tail prevented the centromeric association of Cnp1 protein in Δams2 but not in wild-type (Figure 4), suggesting that Cnp1 may utilize its N-terminal tail for correct centromere targeting and/or formation of stable centromeric nucleosomes at G2 phase. The normal cell cycle progression occurred in wild-type cells expressing the N-terminal tagged GFP-Cnp1 gene (Figure 3), suggesting that the G2-deposition pathway may not be essential when S-deposition is functional. The second gap phase following DNA replication may act as a “rescue” period for kinetochore reassembly prior to cell division when an authentic centromere has been damaged or deregulated. What are the components in the G2-deposition pathway is a significant question to be answered. The increased chromosome mis-segregation in Δams2 mis6 ts double mutant cells suggested that Mis6-Sim4 centromere subcomplex may be involved in the G2-loading pathway as well as the S-phase one (Takahashi et al., 2005). We speculate that both Mis6- and Mis18-complexes are involved in two loading pathways, because Cnp1-signals completely disappear in the mutants (Takahashi et al., 2000; Hayashi et al., 2004; Fujita et al., 2007).

This is the first in vivo report that the replication-uncoupled deposition of Cnp1 occurs under physiological conditions in fission yeast, along with a
description of its functional significance in chromosome segregation. Although
the existence of multiple CENP-A loading pathways was shown to be unlikely in
transformed human cell lines (Jansen et al., 2007), further studies are required to
determine whether the salvage pathway(s) exists in non-transformed normal
cells in higher eukaryotes. The G2-deposition pathway of CENP-A may provide
an evolutionarily conserved safeguard for the maintenance of genome integrity,
and may assist in preventing aneuploid formation during the cell cycle in
non-transformed normal cells.
Acknowledgements

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Online supplemental material

Table S1 lists the genotypes of fission yeast strains used in this study. Figure S1 shows the data quantifying the changes in fluorescence intensity of Cnp1-GFP throughout the cell cycle in wild-type (A) and Δams2 (B). Figure S2 shows that Cnp1 disappeared from the centromeres in Δams2 concomitantly with the S phase-specific protein marker Mrc1. Figure S3 shows that the dissociation of Cnp1 in Δams2 requires completion of DNA replication. Videos 1-3 show the behavior of Cnp1-GFP during the cell cycle in living wild-type (Video 1) or Δams2 cells (Video 2 and 3). Video 4 shows the behaviors of Cnp1-GFP in the mixed culture of wild-type and Δams2 cells. Videos 5-7 show the reloading of Cnp1ts-GFP during the cell cycle in living wild-type (Video 5 for S-deposition and 6 for G2-deposition), Δams2 (Video 7).
Figure Legends

Figure 1. Cnp1-GFP is accumulated on centromeres during G2 and is diminished after S in Δams2. (A) Wild-type (SP92) and Δams2 (SP75) cells expressing Cnp1-GFP were cultured in EMM2 at 33°C. Representative time-lapse images of GFP signals were converted to kymographs. Asterisks and vertical lines indicate the positions of mitotic and septated cells, respectively. Bars indicate 10 μm. (B) Wild-type (SP1055) and Δams2 (SP1698) cells expressing Cnp1-GFP were mixed and cultured in EMM2 at 33°C. Because the wild-type cell carries the Sid4-mRFP gene in the genome, it can be distinguished from the Δams2 cell by detecting mRFP signals before starting the live observation. A series of time-lapse images were taken at 2.5-minute intervals. The graph shows the corresponding intensity of a centromeric GFP-dot in the nucleus plotted as diamonds for wild-type cell and circles for Δams2 cell. The asterisk indicates the period during which the centromeric GFP signals became weak. This period is presumed to be coincident with phase II (from prometaphase to anaphase A), at which centromere clustering becomes loose. After the M phase, we monitored the centromeric signals in one of two daughter cells, which was correctly on the optical plane. The arrow indicates G1/S phase at which a short uptake of Cnp1 incorporation took place. The corresponding movie is shown as Video 4. (C) ChIP assay to estimate the amount of centromere-bound Cnp1 during the cell cycle. Wild-type and Δams2 cells arrested at G1, S, early G2 or late G2 phase were prepared in the presence (gray bars) or absence (white bars) of the native promoter-driven Cnp1-GFP gene at the lys1 locus (see Materials and Methods).
DNA contents of wild-type and Δams2 cells using ChIP assay were estimated by flow cytometry (left). The positions of 1C and 2C peaks are also indicated. DNAs co-precipitated with Cnp1 by using anti-GFP monoclonal antibody (gray bars) or anti-Cnp1 polyclonal antibody (white bars) were quantified by real-time PCR using a cnt2 (central core region of cen2) probe (right). The error bars indicate standard deviation from three independent experiments for early G2 cells or five independent experiments for the others.

Figure 2. Ams2 promotes Cnp1 incorporation, while Hip1 suppresses the cell cycle-independent loading of Cnp1. (A) and (B), Wild-type cells containing the Cnp1<sup>ts</sup>-GFP gene integrated at the lys1 locus (SP1102) were cultured in EMM2 at 36°C, and then shifted to 22°C. Representative time-lapse images of GFP signals incorporated into centromeres in S (A) and G2 (B). The arrowhead indicates the position of the septum. A series of time-lapse images were taken at 1.5-minute intervals. (C) Summary of reloading experiments for Cnp1<sup>ts</sup>-GFP in the wild-type (SP1102), Δams2 (SP1205) or Δhip1 (PHS10) background. Stage I corresponds to M/G1; stage II, G1/S; stage III, S/early G2; stage IV, mid G2; stage V, late G2. The cell cycle distribution (upper; percentages of cell population) and the reloading frequency of Cnp1 (lower; percentages of cells in which Cnp1<sup>ts</sup>-GFP signals appeared at the corresponding stage per total examined cells) are shown. White, black and grey bars represent data for the wild-type (n=240), Δams2 (n=175) and Δhip1 (n=98) backgrounds, respectively.

Figure 3. N-terminal GFP-tagged Cnp1 is functional in normal cell cycle
progression in wild-type cells. (A) Increase in number of the cells in culture in YES at 33°C. Cells expressing the authentic Cnp1 gene (wild-type, SP91), the N-terminal tagged GFP-Cnp1 gene (GFP-Cnp1, SP1769), both the authentic Cnp1 gene and the N-terminal tagged GFP-Cnp1 gene additionally integrated at the \( lys1 \) locus (\( lys1^+ \)::GFP-Cnp1, SP1468), and both of the authentic Cnp1 gene and the C-terminal tagged Cnp1-GFP gene additionally integrated at the \( lys1 \) locus (\( lys1^+ \)::Cnp1-GFP, SP38) were examined. (B) Fivefold serial dilutions of \( 2 \times 10^4 \) cells of wild-type (SP91), GFP-Cnp1 (SP1769), \( lys1^+ \)::GFP-Cnp1 (SP1468), \( lys1^+ \)::Cnp1-GFP (SP38) and \( \Delta \)bub1 (SP1339) were spotted on YES plates in the absence or presence of 6\( \mu \)g/ml CBZ and incubated at 33°C for 3 days. \( \Delta \)bub1 was used as a representative CBZ-sensitive mutant. (C) ChIP assay was performed to confirm that the N-terminal tagged GFP-Cnp1 protein binds correctly to the central core regions of the centromere. Asynchronous cells of GFP-Cnp1 (SP1769) and \( lys1^+ \)::Cnp1-GFP (SP38) strains were prepared by inoculation into YES at 33°C. DNAs co-precipitated with GFP-tagged Cnp1 using anti-GFP antibody or without antibody (data not shown) from cell extracts were quantified by real-time PCR using \( cnt2 \), \( imr1 \), \( otr \) (\( dg \)) and \( act1 \) (background control) probes. (D) The mitotic loss rates of a linear minichromosome, CN2, were examined in wild-type cells (non-tagged Cnp1, SP52) and wild-type cells expressing GFP-Cnp1 (GFP-Cnp1, PHS165) cultured in YES (non-selective medium) at 33°C. Error bars indicate standard deviation from six independent experiments. (E) Cell extracts were prepared from wild-type cells expressing authentic Cnp1 (SP91), GFP-Cnp1 (SP1769), \( lys1^+ \)::GFP-Cnp1 (SP1468) and \( lys1^+ \)::Cnp1-GFP (SP92), and \( \Delta \)ams2 cells expressing authentic Cnp1 (YTP155), \( lys1^+ \)::GFP-Cnp1 (SP1637) and
lys1::Cnp1-GFP (SP75) cultured in YES at 33°C. Levels of endogenous and exogenous Cnp1 protein expression were examined by immunoblotting using anti-GFP and anti-Cnp1 antibodies. α-tubulin detected by TAT1 antibody was used as a loading control.

Figure 4. The intact N-terminal tail plays a role in Ams2-dependent retention of Cnp1 at the centromere. (A) N-terminally tagged Cnp1 exerts synthetic lethal effect on Ams2-depletion. Wild-type cells expressing GFP-Cnp1 (SP1769) and lys1::GFP-Cnp1 (SP1468) and Δams2 cells expressing GFP-Cnp1 (cell samples were prepared from germinated spores) and lys1::GFP-Cnp1 (SP1637) were cultured in EMM2 at 26°C. Representative images of GFP signals and DAPI-staining are shown. Arrowheads indicate the chromosome missegregation observed in Δams2 cells expressing GFP-Cnp1. (B) The centromeric localization activities of N-terminally deleted derivatives are summarized as indicated by ++ and + for strong and weak centromere-like signals, respectively, and – for non-centromeric, dispersed nuclear (nuc) or cellular (cell) signals.

Figure 5. G2 phase acts as the second chance for Cnp1 incorporation. (A) Nuclear morphology (YTP370) stained with DAPI and frequencies of binucleate cells with asymmetric nuclei of p<sup>amt1</sup>-ams2 (ams2-shut-off strain, YTP366) or p<sup>amt1</sup>-ams2 wee1-50 double (YTP370) mutant cultured in EMM2 in the absence (ams2-ON) or presence (ams2-OFF) of thiamine at 33°C for 4 h. The error bars indicate standard deviations from three independent experiments. Arrowheads indicate cells with large and small daughter nuclei. Septated cells with a single
nucleus (asterisks) were counted as cells with asymmetric nuclei. (B) Synthetic lethality of Ams2-null with wee1-50 mutation. Colony formation of wild-type (SP143), wee1-50 (YTP374), p<sup>nmkt81</sup>-ams2 (YTP366) and p<sup>nmkt81</sup>-ams2 wee1-50 (YTP370) on EMM2 in the absence (ams2-ON) or presence (ams2-OFF) of thiamine at 33°C for 4 days. (C) Cnp1-GFP localization and DAPI-staining of representative mitotic wild-type (WT, SP92), p<sup>nmkt81</sup>-ams2 (ams2-OFF, YTP389), wee1-50 (YTP393) and p<sup>nmkt81</sup>-ams2 wee1-50 (ams2-OFF wee1-50, YTP390) cells expressing Cnp1-GFP. Cells were cultured in EMM2 at 33°C for 3 h after addition of thiamine. (D) Colony formation of cdc25-22 (YTP379), cdc25-22 Δams2 (YTP354) and Δams2 (YTP155) on YES plate at 26°C for 5 days. Bar, 1 cm. The average doubling times of each strain cultured in YES at 26°C are also shown with standard deviations from four independent experiments.
References


Figure 1

A.

Wild-type and Δams2 images showing a comparison of DNA replication patterns.

B.

Graph comparing relative intensity over time for wild-type and Δams2 samples. The graph illustrates a significant difference in replication dynamics between the two conditions.

C.

Analysis of DNA content histograms showing the relative intensity of early G2, late G2, G1, and S phases for wild-type and Δams2 samples. The histograms provide a quantitative comparison of DNA content changes.

Takayama et. al Fig. 1
Takayama et al. Fig. 3
B

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Takayama et. al Fig.4