Biphasic Incorporation of Centromeric Histone CENP-A in Fission Yeast

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Submitted May 29, 2007; Revised November 12, 2007; Accepted November 27, 2007

Monitoring Editor: Kerry Bloom

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 Δ ams2, CENP-A fails to retain during S, but it reaccumulates onto centromeres via the G2 deposition pathway, which is down-regulated by Hip1, a homologue of HIRA histone chaperon. Reducing the length of G2 in Δ ams2 results in failure of CENP-A accumulation, leading to chromosome missegregation. N-terminal green fluorescent protein-tagging reduces the centromeric association of CENP-A, causing cell death in Δ ams2 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 manner 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 that 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, 2007). Because 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 before 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), the 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 used for CENP-A incorporation remains controversial. During S phase, canonical core histones have been suggested to be deposited into

duplicated DNAs in a semiconservative manner (Tagami *et al.*, 2004; Natsume *et al.*, 2007). Experiments using fluorescence recovery after photobleaching 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 Δ ams2 cells are defective in the retention of Cnp1, *S. pombe* CENP-A, at S phase, but they are able to survive through Cnp1 incorporation using the replication-independent pathway at G2. Our observations indicated that the G2 deposition of Cnp1 is mechanistically 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 Supplemental Table S1. Anti-Cnp1 polyclonal antibody was generated by immunizing rabbits with glutathione S transferase-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-green fluorescent protein (GFP) gene, the open reading frame (ORF) of the deleted derivatives of Cnp1 gene was amplified by PCR and inserted into the *Sall*–*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 *nmf41* promoter. Polymerase chain reaction (PCR) primer sequences used for the PCR ampli-

This article was published online ahead of print in *MBC in Press* (<http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E07-05-0504>) on December 12, 2007.

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Abbreviations used: ChIP, chromatin immunoprecipitation.

fication were as follows: full length, 5'-GTCGACATGGCAAAGAAATC-3'/5'-GCGGCCGCTAGCACCACGAATCC-3'; D5, 5'-GTCGACATGGCTGAG-CCTGG-3'/5'-GCGGCCGCTAGCACCACGAATCC-3'; D10, 5'-GTCGACA-TGGATCCTATTCAC-3'/5'-GCGGCCGCTAGCACCACGAATCC-3'; D15, 5'-GTCGACATGCCACGTAAG-3'/5'-GCGGCCGCTAGCACCACGAATCC-3'; and D20, 5'-GTCGACATGTATCGTCCAGGTAC-3'/5'-GCGGCCGCTAGCACCACGAATCC-3'.

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 enhanced green fluorescent protein gene (Clontech Laboratories, Heidelberg, Germany) amplified by PCR by using primers containing HindIII and BamHI sites was inserted into the cloning sites in frame. A *cnp1-1^{ts}* strain (SP1786, *ura4 Δ cnp1::ura4⁺lys1⁺::cnp1-1*) was transformed with PS1, and the transformants were grown at 22°C (permissive temperature for *cnp1-1*) on Edinburgh minimal medium (EMM)2 plates in the presence of 1 mg/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 Figures 3 and 4. To generate the *lys1⁺::GFP-Cnp1* strain (SP1468), a 2.4-kb KpnI-EcoRI fragment containing the GFP-Cnp1 gene was subcloned into the plasmid pTK2 designed for integration of the inserted DNAs into the *lys1⁺* locus. A *lys1⁻* 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⁺* locus in the transformants was confirmed by genomic Southern analysis.

Microscopy

For 4,6-diamidino-2-phenylindole (DAPI) staining in Figure 5, cells were cultured in EMM2 containing the appropriate supplements with or without 2 μ M 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 100 $\times$ 1.45 numerical aperture α -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 an ASMDW live cell imaging system microscope with a 100 $\times$ 1.40 numerical aperture ACX PL Apo objective (Leica Microsystems, Wetzlar, Germany). Images collected every 0.3 μ m along the z-axis were processed with the nonblind three-dimensional deconvolution algorithm by using ASMDW software. The projected images were converted to kymographs with MetaMorph software (Molecular Devices, Sunnyvale, CA). The fluorescence intensity of the centromeric GFP-dots and that of the nuclear GFP background were calculated by Image Quant IL (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom) after the background (signals outside of the cell) titration. For the experiments shown in Figure 2, 513 videos in total were recorded and analyzed individually. Cells were classified into five cell cycle stages: stage I, unseptated binucleate cells (M/G1); stage II, septated binucleate cells (G1/S); and stages III-V, single nuclear cells <7.1 μ m (III, S/early G2), 7.1-10.5 μ m (IV, mid-G2), or >10.5 μ m (V, late G2) in length. Because the average septated cell length of Δ hip1 was 36% longer than that of wild-type controls, we used the following values as the criteria of G2 classification for Δ hip1: stage III-V, single nuclear cells <9.6 μ m (III), 9.6-14.3 μ m (IV), or >14.3 μ m (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 they were then shifted to 36°C for 3.5 h (*cdc25-22 lys1⁺::Cnp1-GFP*; SP1234, *cdc25-22*; YTP379, *cdc25-22* Δ ams2 *lys1⁺::Cnp1-GFP*; SP1751 and *cdc25-22* Δ ams2; 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* Δ ams2 *lys1⁺::Cnp1-GFP*; SP1699, *cdc10-129* Δ ams2; SP2960 for G1, and *cdc22-C11* Δ ams2 *lys1⁺::Cnp1-GFP*; SP1704, *cdc22-C11* Δ ams2; SP2963 for S). Wild-type (SP91), *lys1⁺::Cnp1-GFP* (SP92), Δ ams2 (YTP155), and Δ ams2 *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). ChIP assays were performed using anti-GFP monoclonal antibody (mAb) (Roche, Indianapolis, IN) and Dynabeads anti-mouse immunoglobulin (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 im-

munoprecipitated fractions were analyzed by real-time PCR by using an ABI 7000 Sequence Detection System and Power SYBR Green PCR master mix (Applied Biosystems, Foster City, CA). The ratios of ChIP signals for *cnp1* in the Δ ams2 background were quantified as intensities relative to those in the *ams2⁺* background. In all samples, intensities of ChIP signals for *otr* (Saitoh *et al.*, 1997) and *act1* probes (background controls) were <3.6 and 0.2% of those for the *cnp1* probe, respectively. PCR primer sequences were as follows: *cnp1*, 5'-AAAGCAAACAGCAGTAACCTGTAA-3'/5'-TGCCTCTTATATGCGGCTTA-3'; *imr1*, 5'-CCTTTACTGGAAATGTGCG-3'/5'-GCTGAGGCTAAGTATCTGTT-3'; and *otr* (*dg*), 5'-CATGGAACACTACGTCAGGAGTGG-3'/5'-TGCCTGTTCACITATCTAATTCG-3'; and *act1*, 5'-CTTCTACAACGAGCTTC-TGTGTG-3'/5'-GAGTCATCTTCTACGGTTGGAT-3'.

RESULTS

Cell Cycle-dependent Centromeric Localization of Cnp1 in Δ ams2

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 by using wild-type or Δ ams2 carrying the C-terminal GFP-tagged Cnp1 gene driven by the native promoter (Figure 1, A and B, Supplemental Figure S1, and Supplemental Videos 1-4). In Δ ams2, we found that Cnp1-GFP is located on centromeres in a cell cycle-dependent manner. 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 Δ ams2 cells (Supplemental 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 Δ ams2 in the same frame. In the image of both of the wild-type and the Δ ams2 cell, the centromere fluorescence of Cnp1 signals seemed to rapidly drop as chromosomes segregate (Figure 1B and Supplemental Figure S1, asterisks), but it 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 Δ ams2 cell, a short uptake of Cnp1 incorporation occurred at G1/S phase (Figure 1B and Supplemental Figure S1B, arrows). Because Cnp1 is transcribed at G1/S phase before 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 Δ ams2 cells.

We performed 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 Δ ams2 background was almost equivalent to that in wild-type controls, whereas

Figure 1. Cnp1-GFP is accumulated on centromeres during G2, and it is diminished after S in Δ ams2. (A) Wild-type (SP92) and Δ ams2 (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, 10 μ m. (B) Wild-type (SP1055) and Δ ams2 (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 Δ ams2 cell by detecting monomeric red fluorescent protein signals before starting the live observation. A series of time-lapse images were taken at 2.5-min 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 Δ ams2 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 Δ ams2 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 Δ ams2 cells by using ChIP assay were estimated by flow cytometry (left). The positions of 1C and 2C peaks are also indicated. DNAs coprecipitated with Cnp1 by using anti-GFP mAb (gray bars) or anti-Cnp1 polyclonal antibody (white bars) were quantified by real-time PCR by using a *cnt2* (central core region of *cen2*) probe (right). The error bars indicate SD from three independent experiments for early G2 cells or five independent experiments for the others.

those at G1, early S, and early G2 phases were reduced to 49, 27, and 5%, respectively. In the Δ ams2 background, Cnp1-GFP was associated correctly with the central centromere region (*cnt*). These observations indicate that, in Δ ams2, the centromeric Cnp1-GFP is diminished during S phase, and then it disappears. Inhibition of DNA synthesis by addition of hydroxyurea (HU) blocked the disappearance of Cnp1-GFP signals in Δ ams2 (Supplemental Figure S3), indicating that the dissociation of Cnp1-GFP in Δ ams2 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 nontagged Cnp1 that is expressed from the authentic locus. The total amount of centromere-bound Cnp1 in Δ ams2 relative to that in wild-type controls was reduced to 49% in early S and 46% in early G2 phase, whereas 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 it 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 Δ ams2 cells highlights substantial Ams2-independent Cnp1 localization activity during the later half of G2 (Figure 1 and Supplemental 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 Cnp1^{ts} protein (Chen *et al.*, 2003a). The Cnp1^{ts}-GFP gene driven by the native promoter was integrated into the genome of wild-type or Δ ams2 cells, and the resultant cells carried both the Cnp1^{ts} gene and the authentic gene, but only the ts protein was visible by GFP-tagging. The Cnp1^{ts} protein with a mutation (L87Q) in the middle of the α 2 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^{ts}-GFP dynamics after shifting from 36°C to a low temperature of 22°C. For comparison between wild-type and Δ ams2, the cells were classified into five morphological categories (stages I–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 Δ ams2 cell culture was roughly comparable with that of wild-type controls (Figure 2C, top). Mass analyses of Cnp1 reloading assay indicated that Cnp1^{ts}-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, bottom: stages II–III, Supplemental Video 5). In the remaining 24.6% of the wild-type cells, Cnp1^{ts}-GFP accumulated at late G2 (Figure 2, B and C, bottom: stage V, Supplemental 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^{ts}-GFP incorporation across the cell cycle, at S and late G2 phases, with no Cnp1^{ts}-GFP incorporation in M/G1 (Figure 2C, bottom: stage I) or mid-G2 (stage IV).

In contrast, in Δ ams2, reloading of Cnp1^{ts}-GFP around S phase was reduced to 17.7% (31 of 175 cells examined; Figure 2C, bottom: stages II and III), whereas that during late G2 increased to 82.3% (stage V). In most cases in Δ ams2, Cnp1^{ts}-GFP occurred as dispersed signals in the nucleus but failed to accumulate into centromeres at S phase, which was then reloaded at late G2 (Supplemental 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 Δ ams2 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 Δ ams2 cells in which pre-existing Cnp1 is retained on centromeres.

Hip1-dependent Repression of Cnp1 Deposition during G2 Phase

The above-mentioned observation raises questions regarding the components of the G2 deposition pathway. We examined whether the fission yeast homologue 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 Δ hip1 cells (4.4 h) was 1.5 times longer than that of wild-type cells (3.0 h) in YES at 26°C, the cell cycle progression of Δ hip1 cells seems comparable with that of wild-type by using the five morphological categories; judging from the timing of the appearance of Mrc1 protein, the S phase in Δ hip1 cells seemed to occur during the septum formation at same as in wild-type and Δ ams2 (data not shown). This shows that the G2 phase starts with the normal timing in Δ hip1 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 Δ hip1 background (data not shown). However, we found that Cnp1^{ts} reloading occurred throughout G2 in cells lacking Hip1 (Figure 2C). In addition to Cnp1^{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* homologue of HIRA protein. Alternatively, Hip1 may promote the refolding, reassembly of Cnp1^{ts} protein, or both 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 Δ ams2 cells did not suppress the growth retardation, but rather it showed the synthetic growth defect (data not shown), suggesting that Hip1 and Ams2 have the additional roles in cell growth besides regulating Cnp1 loading.

Figure 2. Ams2 promotes Cnp1 incorporation, whereas Hip1 suppresses the cell cycle-independent loading of Cnp1. (A and B) Wild-type cells containing the Cnp1^{ts}-GFP gene integrated at the *lys1* locus (SP1102) were cultured in EMM2 at 36°C, and then they were 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-min intervals. Bar, 10 μ m. (C) Summary of reloading experiments for Cnp1^{ts}-GFP in the wild-type (SP1102), Δ ams2 (SP1205), or Δ hip1 (PHS10) background. Stage I corresponds to M/G1; stage II, G1/S; stage III, S/early G2; stage IV, mid-G2; and stage V, late G2. The cell cycle distribution (top; percentages of cell population) and the reloading frequency of Cnp1 (bottom; percentages of cells in which Cnp1^{ts}-GFP signals occurred at the corresponding stage per total examined cells) are shown. White, black, and gray bars represent data for the wild-type ($n = 240$), Δ ams2 ($n = 175$), and Δ hip1 ($n = 98$) 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 2×10^4 cells of wild-type (SP91), GFP-Cnp1 (SP1769), *lys1⁺::GFP-Cnp1* (SP1468), *lys1⁺::Cnp1-GFP* (SP38), and Δ bub1 (SP1339) were spotted on YES plates in the absence or presence of 6 μg/ml CBZ and incubated at 33°C for 3 d. Δ 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 coprecipitated with GFP-tagged Cnp1 by using anti-GFP antibody or without antibody (data not shown) from cell extracts were quantified by real-time PCR by 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 (nontagged Cnp1, SP52) and wild-type cells expressing GFP-Cnp1 (GFP-Cnp1, PHS165) cultured in YES (nonselective medium) at 33°C. Error bars indicate SD 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 Δ 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 by using anti-GFP and anti-Cnp1 antibodies. α -Tubulin detected by TAT1 antibody was used as a loading control.

N-terminal GFP-tagged Cnp1 Is Functional in Wild Type but Not in Δ ams2

Because 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 we 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; 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 colocalization 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, whereas that of GFP-Cnp1 expressed from the *lys1* locus or from the native locus was comparable with that of authentic Cnp1 protein (Figure 3E). The level of expression of each tagged Cnp1 protein in the Δ ams2 background was comparable with that in wild-type cells (Figure 3E).

The above-mentioned 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 microcolonies but soon ceased growing. Microscopic observation of the microcolonies 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

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 Δ ams2 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 Δ ams2 cells expressing GFP-Cnp1. Bar, 10 μ m. (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 noncentromeric, dispersed nuclear (nuc) or cellular (cell) signals.

by a small decrease in GFP-Cnp1 protein in the Δ ams2 background. Additional integration of the GFP-Cnp1 gene at the *lys1* locus in Δ ams2 cells resulted in recovery of cell viability to the level in Δ ams2 cells expressing nontagged Cnp1 (data not shown). However, the centromeric localization activity of GFP-Cnp1 was clearly defective (Figure 4A). Therefore, the growth arrest observed in Δ ams2 spores expressing GFP-Cnp1 is not due to a dominant-negative effect of GFP-Cnp1 on Ams2-deletion, but it is presumably due to the failure of GFP-Cnp1 retention at centromeres in Δ ams2 cells. One interesting possibility suggested by the results shown in Figures 3 and 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 Δ ams2 cells expressing N-terminal tagged GFP-Cnp1^{ts} protein expressed from the *lys1* locus for reloading assay. However, because the centromeric localization activity of GFP-Cnp1^{ts} protein coexpressed 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-mentioned 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-terminally deleted derivatives of Cnp1-GFP (Figure 4B). Overexpressed full-length Cnp1-GFP and D5 derivative accumulated on the centromeres in both wild-type and Δ ams2 cells, whereas 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, although D10 derivatives were imported into the nucleus both in wild-type cells and Δ ams2 cells, they were located on the centromeres in wild-type cells but not in Δ ams2 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-mentioned 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 an *S. pombe* cell has evolved two distinct deposition pathways. Δ ams2 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 they grew normally with equal chromosome segregation in M phase (Figure 5A, *ams2*-ON). In contrast, the cells exhibited a high frequency of unequal chromosome segregation with the *wee* phenotype (Figure 5A, *ams2*-OFF), and they did not survive when Ams2 was repressed (Figure 5B). The centromeric localization of Cnp1-GFP was markedly reduced in *ams2*-deficient *wee1-50* mutant in comparison with *wee1-50*, *ams2*-deficient or wild-type controls (Figure 5C). In contrast, Δ ams2 *cdc25-22* double mutant with prolonged G2 phase (Hayles and Nurse, 1992) showed much better growth than the single Δ 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

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. Although 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^{ts}) 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 Δ ams2 cells, whereas overproduction of histone H3, which competes with Cnp1 for H4 binding, is toxic to Δ ams2 cell growth (Chen *et al.*,

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^{mt81}-ams2* (*ams2*-shut-off strain, YTP366) or *p^{mt81}-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. Bar, 10 μ m. (B) Synthetic lethality of Ams2-null with *wee1-50* mutation. Colony formation of wild-type (SP143), *wee1-50* (YTP374), *p^{mt81}-ams2* (YTP366), and *p^{mt81}-ams2 wee1-50* (YTP370) on EMM2 in the absence (*ams2*-ON) or presence (*ams2*-OFF) of thiamine at 33°C for 4 d. (C) Cnp1-GFP localization and DAPI staining of representative mitotic wild-type (WT, SP92), *p^{mt81}-ams2* (*ams2*-OFF, YTP389), *wee1-50* (YTP393), and *p^{mt81}-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. Bar, 10 μ m. (D) Colony formation of *cdc25-22* (YTP379), *cdc25-22 Δams2* (YTP354), and *Δams2* (YTP155) on YES plate at 26°C for 5 d. 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.

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. Because the supply of histone H4 in Δ *ams2* 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 al.*, 2003b), or other as yet unidentified transcriptional targets of Ams2 may be important for the formation/retention of Cnp1-containing nucleosomes at S phase, or a combination.

Because the intracellular levels of histone proteins are not markedly affected in Δ *ams2* (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. Because 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 rechromatinized after 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 Δ *ams2* 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 it 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 Δ *ams2* cells revealed that a short uptake of Cnp1 deposition in G1 and/or early S phase exists (Figure 1B and Supplemental 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 Δ *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).

Evolutional Conservation of CENP-A Dynamics

The cell cycle timing of CENP-A loading seems 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 by 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 α , hMis18 β , 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 noncentromeric Cse4 during S phase (Bloom, 2007). In the early *Drosophila* blastoderm embryo, in <2 min after photobleaching, >100% of the initial fluorescent intensity of GFP-tagged centromere identifier (CID), the *Drosophila* homologue 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

Because the *wee1* mutant with a shortened G2 phase showed no apparent defects in Cnp1 localization (Figure 5C), S deposition seems 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 Δ ams2 but not in wild type (Figure 4), suggesting that Cnp1 may use 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 after DNA replication may act as a "rescue" period for kinetochore reassembly before 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 missegregation in Δ ams2 mis6 ts double mutant cells suggested that Mis6-Sim4 centromere subcomplex may be involved in the G2 loading pathway and the S phase pathway (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 nontransformed 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 it may assist in preventing aneuploid formation during the cell cycle in nontransformed normal cells.

ACKNOWLEDGMENTS

We thank Prof. M. Yanagida for providing materials used in this study, Prof. K. Gull for the TAT1 antibody, and Dr. K. Tanaka for the *wee1-50* mutant cells. This work was supported by grants-in-aid for scientific research on priority areas, nuclear dynamics; funds from Ministry of Education, Culture, Sports, Science and Technology; a grant from the Toray Science Foundation; and a grant from Time's Arrow and Biosignaling, Precursory Research for Embryonic Science and Technology, Japan Science and Technology Agency.

REFERENCES

- Ahmad, K., and Henikoff, S. (2001). Centromeres are specialized replication domains in heterochromatin. *J. Cell Biol.* 153, 101–110.
- Ahmad, K., and Henikoff, S. (2002). The histone variant H3.3 marks active chromatin by replication-independent nucleosome assembly. *Mol. Cell.* 9, 1191–1200.
- Amor, D. J., and Choo, K. H. (2002). Neocentromeres: role in human disease, evolution, and centromere study. *Am. J. Hum. Genet.* 71, 695–714.
- Black, B. E., Foltz, D. R., Chakravarthy, S., Luger, K., Woods, V. L., Jr., and Cleveland, D. W. (2004). Structural determinants for generating centromeric chromatin. *Nature* 430, 578–582.
- Black, B. E., Jansen, L. E., Maddox, P. S., Foltz, D. R., Desai, A. B., Shah, J. V., and Cleveland, D. W. (2007). Centromere identity maintained by nucleosomes assembled with histone H3 containing the CENP-A targeting domain. *Mol. Cell.* 25, 309–322.
- Blackwell, C., Martin, K. A., Greenall, A., Pidoux, A., Allshire, R. C., and Whitehall, S. K. (2004). The *Schizosaccharomyces pombe* HIRA-like protein Hip1 is required for the periodic expression of histone genes and contributes to the function of complex centromeres. *Mol. Cell. Biol.* 24, 4309–4320.
- Bloom, K. (2007). Centromere dynamics. *Curr. Opin. Genet. Dev.* 17, 151–156.
- Chen, E. S., Saitoh, S., Yanagida, M., and Takahashi, K. (2003a). A cell cycle-regulated GATA factor promotes centromeric localization of CENP-A in fission yeast. *Mol. Cell.* 11, 175–187.
- Chen, E. S., Yanagida, M., and Takahashi, K. (2003b). Does a GATA factor make the bed for centromeric nucleosomes? *Cell Cycle* 2, 277–278.
- Cleveland, D. W., Mao, Y., and Sullivan, K. F. (2003). Centromeres and kinetochores: from epigenetics to mitotic checkpoint signaling. *Cell* 112, 407–421.

- Collins, K. A., Furuyama, S., and Biggins, S. (2004). Proteolysis contributes to the exclusive centromere localization of the yeast Cse4/CENP-A histone H3 variant. *Curr. Biol.* 14, 1968–1972.
- Fujita, Y., Hayashi, T., Kiyomitsu, T., Toyoda, Y., Kokubu, A., Obuse, C., and Yanagida, M. (2007). Priming of centromere for CENP-A recruitment by human hMis18alpha, hMis18beta, and M18BP1. *Dev. Cell.* 12, 17–30.
- Hayashi, T., Fujita, Y., Iwasaki, O., Adachi, Y., Takahashi, K., and Yanagida, M. (2004). Mis16 and Mis18 are required for CENP-A loading and histone deacetylation at centromeres. *Cell* 118, 715–729.
- Hayles, J., and Nurse, P. (1992). Genetics of the fission yeast *Schizosaccharomyces pombe*. *Annu. Rev. Genet.* 26, 373–402.
- Henikoff, S., and Dalal, Y. (2005). Centromeric chromatin: what makes it unique? *Curr. Opin. Genet. Dev.* 15, 177–184.
- Holmes, S. G., and Mitchell Smith, M. (2001). Replication of minichromosomes in *Saccharomyces cerevisiae* is sensitive to histone gene copy number and strain ploidy. *Yeast* 18, 291–300.
- Jans, D. A., Xiao, C. Y., and Lam, M. H. (2000). Nuclear targeting signal recognition: a key control point in nuclear transport? *Bioessays* 22, 532–544.
- Jansen, L. E., Black, B. E., Foltz, D. R., and Cleveland, D. W. (2007). Propagation of centromeric chromatin requires exit from mitosis. *J. Cell Biol.* 176, 795–805.
- Karpen, G. H., and Allshire, R. C. (1997). The case for epigenetic effects on centromere identity and function. *Trends Genet.* 13, 489–496.
- Maddox, P. S., Hyndman, F., Monen, J., Oegema, K., and Desai, A. (2007). Functional genomics identifies a Myb domain-containing protein family required for assembly of CENP-A chromatin. *J. Cell Biol.* 176, 757–763.
- Maruyama, S., Kuroiwa, H., Miyagishima, S. Y., Tanaka, K., and Kuroiwa, T. (2007). Centromere dynamics in the primitive red alga *Cyanidioschyzon mero-lae*. *Plant J.* 49, 1122–1129.
- Meeks-Wagner, D., and Hartwell, L. H. (1986). Normal stoichiometry of histone dimer sets is necessary for high fidelity of mitotic chromosome transmission. *Cell* 44, 43–52.
- Mellone, B. G., and Allshire, R. C. (2003). Stretching it: putting the CEN(P-A) in centromere. *Curr. Opin. Genet. Dev.* 13, 191–198.
- Nabeshima, K., Nakagawa, T., Straight, A. F., Murray, A., Chikashige, Y., Yamashita, Y. M., Hiraoka, Y., and Yanagida, M. (1998). Dynamics of centromeres during metaphase-anaphase transition in fission yeast: Dis1 is implicated in force balance in metaphase bipolar spindle. *Mol. Biol. Cell* 9, 3211–3225.
- Natsume, R., Eitoku, M., Akai, Y., Sano, N., Horikoshi, M., and Senda, T. (2007). Structure and function of the histone chaperone CIA/ASF1 complexed with histones H3 and H4. *Nature* 446, 338–341.
- Pearson, C. G., Yeh, E., Gardner, M., Odde, D., Salmon, E. D., and Bloom, K. (2004). Stable kinetochore-microtubule attachment constrains centromere positioning in metaphase. *Curr. Biol.* 14, 1962–1967.
- Pidoux, A. L., Richardson, W., and Allshire, R. C. (2003). Sim 4, a novel fission yeast kinetochore protein required for centromeric silencing and chromosome segregation. *J. Cell Biol.* 161, 295–307.
- Ray-Gallet, D., Quivy, J. P., Scamps, C., Martini, E. M., Lipinski, M., and Almouzni, G. (2002). HIRA is critical for a nucleosome assembly pathway independent of DNA synthesis. *Mol. Cell* 9, 1091–1100.
- Rustici, G., Mata, J., Kivinen, K., Lio, P., Penkett, C. J., Burns, G., Hayles, J., Brazma, A., Nurse, P., and Bahler, J. (2004). Periodic gene expression program of the fission yeast cell cycle. *Nat. Genet.* 36, 809–817.
- Saitoh, S., Ishii, K., Kobayashi, Y., and Takahashi, K. (2005). Spindle checkpoint signaling requires the mis6 kinetochore subcomplex, which interacts with mad2 and mitotic spindles. *Mol. Biol. Cell* 16, 3666–3677.
- Saitoh, S., Takahashi, K., and Yanagida, M. (1997). Mis6, a fission yeast inner centromere protein, acts during G1/S and forms specialized chromatin required for equal segregation. *Cell* 90, 131–143.
- Schuh, M., Lehner, C. F., and Heidmann, S. (2007). Incorporation of *Drosophila* CID/CENP-A and CENP-C into centromeres during early embryonic anaphase. *Curr. Biol.* 17, 237–243.
- Shelby, R. D., Monier, K., and Sullivan, K. F. (2000). Chromatin assembly at kinetochores is uncoupled from DNA replication. *J. Cell Biol.* 151, 1113–1118.
- Sullivan, B., and Karpen, G. (2001). Centromere identity in *Drosophila* is not determined in vivo by replication timing. *J. Cell Biol.* 154, 683–690.
- Tagami, H., Ray-Gallet, D., Almouzni, G., and Nakatani, Y. (2004). Histone H3.1 and H3.3 complexes mediate nucleosome assembly pathways dependent or independent of DNA synthesis. *Cell* 116, 51–61.
- Takahashi, K., Chen, E. S., and Yanagida, M. (2000). Requirement of Mis6 centromere connector for localizing a CENP-A-like protein in fission yeast. *Science* 288, 2215–2219.
- Takahashi, K., Murakami, S., Chikashige, Y., Funabiki, H., Niwa, O., and Yanagida, M. (1992). A low copy number central sequence with strict symmetry and unusual chromatin structure in fission yeast centromere. *Mol. Biol. Cell* 3, 819–835.
- Takahashi, K., Takayama, Y., Masuda, F., Kobayashi, Y., and Saitoh, S. (2005). Two distinct pathways responsible for the loading of CENP-A to centromeres in the fission yeast cell cycle. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 360, 595–606; discussion 606–597.
- Takahashi, K., Yamada, H., and Yanagida, M. (1994). Fission yeast minichromosome loss mutants mis cause lethal aneuploidy and replication abnormality. *Mol. Biol. Cell* 5, 1145–1158.
- Takayama, Y., and Takahashi, K. (2007). Differential regulation of repeated histone genes during the fission yeast cell cycle. *Nucleic Acids Res.* 35, 3223–3237.