Cell cycle-dependent deposition of CENP-A requires the Dos1/2–Cdc20 complex

Marlyn Gonzalez, Haijin He, Siyu Sun, Chen Li, and Fei Li[1]

Department of Biology, New York University, New York, NY 10003

Edited by Jasper Rine, University of California, Berkeley, CA, and approved November 28, 2012 (received for review August 29, 2012)

Centromeric histone CENP-A, a variant of canonical histone H3, plays a central role in proper chromosome segregation. Loading of CENP-A at centromeres is cell cycle-regulated: parental CENP-A is deposited at centromeres during S phase, whereas newly synthesized CENP-A is deposited during later stages of the cell cycle. The mechanisms involved in deposition of CENP-A at centromeres during S phase remain poorly understood. In fission yeast, loading of CENP-A during S phase is regulated by the GATA-type factor, Ams2. Here we show that the Dos1/2-Cdc20 complex, previously characterized as a silencing complex essential for inheritance of H3K9 methylation during S phase, is also required for localization of CENP-A^{cnp1} at centromeres at this stage. Disruption of Dos1 (also known as Raf1/Clr8/Cmc1), Dos2 (also known as Raf2/Clr7/Cmc2), or Cdc20, a DNA polymerase epsilon subunit, results in dissociation of CENP-A from centromeres and mislocalization of the protein to noncentromeric sites. All three mutants display spindle disorganization and mitotic defects. Inactivation of Dos1 or Cdc20 also results in accumulation of noncoding RNA transcripts from centromeric cores, a feature common to mutants affecting kinetochore integrity. We further find that Dos1 physically associates with Ams2 and is required for the association of Ams2 with centromeric cores during S phase. Finally, we show that Dos2 associates with centromeric cores during S phase and that its recruitment to centromeric cores depends on Cdc20. This study identifies a physical link between DNA replication and CENP-A assembly machinery and provides mechanistic insight into how CENP-A is faithfully inherited during S phase.

epigenetics | heterochromatin

Centromeres, and the kinetochores that assemble on them, mediate proper chromosome segregation during cell division (1). Although centromere function is conserved among eukaryotes, the underlying DNA sequences vary greatly in size and composition across species (1, 2). CENP-A, a centromere-specific histone 3 (H3) variant, is the most favored candidate for the epigenetic mark responsible for specifying centromere identity during cell division (1). CENP-A replaces canonical histone H3 at centromeres and serves as the foundation for kinetochore assembly (1, 2). Defects in CENP-A regulation result in chromosome mis-segregation and reduced growth (3). Assembly of CENP-A at centromeres is cell cycle-regulated. During DNA replication, centromeric nucleosomes are disassembled ahead of replication forks (4). Studies in both *Drosophila* and human cells revealed that preexisting CENP-A is redistributed evenly between daughter centromeres following DNA replication during S phase, whereas newly made CENP-A is loaded at later stages of the cell cycle (2, 5, 6). Recent findings identify the histone chaperone HJURP (Holliday junction recognition protein)/Scm3 as required for recruitment of newly synthesized CENP-A to centromeres in a DNA replication-independent manner (7–10). However, little is known about how DNA replication and CENP-A assembly factors coordinate to promote deposition of CENP-A at centromeres during S phase.

Schizosaccharomyces pombe contains "regional" centromeres, each of which contains multiple microtubule attachment sites. Its CENP-A homolog, Cnp1 (CENP-A^{cnp1}), resides within a central core domain (*cnt*) of ~12 kb in length that is flanked by highly condensed heterochromatin (11). In contrast, the much smaller "point" centromeres of budding yeast (*Saccharomyces cerevisiae*) form a single microtubule attachment per chromosome and are not flanked by heterochromatin (1). The structure and biology of regional centromeres is highly conserved among eukaryotes, including mammals (12). In *S. pombe,* deposition of CENP-A^{cnp1} takes place during the S and G2 phases of the cell cycle (13, 14). Two distinct pathways have been shown to regulate CENP-A^{cnp1} deposition at centromeres: the Mis6- and Ams2-dependent pathways (13). Mis6 is a highly conserved protein that is essential for viability and has been shown to be required for deposition of CENP-A^{cnp1} at centromeres during the S and G2 phases of the cell cycle (13, 15). The human homolog of Mis6, CENP-I, forms a complex with CENP-H, and this complex is important for directing CENP-A^{cnp1} deposition at centromeres (16). Ams2 is a cell cycle-regulated GATA-type factor and plays a key role in the deposition of CENP-A^{cnp1} at centromeres during S phase. Although Ams2 is not essential for viability, *ams2*Δ mutants fail to properly incorporate CENP-A at centromeres during S phase and are compromised for growth (17). The residual growth observed in these mutants is believed to result from salvage mechanisms, including the Mis6-dependent pathway, that are activated during G2 and enable enough CENP-A loading at centromeres to partially restore growth (13, 14).

As in mammalian cells, centromeres in fission yeast are embedded in heterochromatin, rich in tandem DNA repeats. In fission yeast, DNA repeats most proximal to centromeric cores are referred to as innermost repeats (*imr*), which are flanked by more distal outer repeats (*otr*) (11). These pericentromeric repeat regions are enriched in methylation of H3 at lysine 9 (H3K9me), a hallmark for heterochromatin (11). This modification is catalyzed by Clr4, a member of the SUV39 family of histone methyltransferases. The H3K9me mark is recognized and bound by Swi6, a homolog of metazoan Heterochromatin Protein 1 (HP1) (18). During early S phase, heterochromatin is briefly transcribed and the transcripts are processed into small interfering RNAs (siRNAs) by RNA interference (RNAi) machinery (18, 19). These siRNAs, together with silencing factors, including Rik1, Dos1, Dos2, and Lid2, mediate Clr4's H3K9 methylation activity (20–25). Recently, we found that the DNA polymerase (Pol) epsilon subunit, Cdc20, interacts with the Dos1–Dos2 silencing complex to promote heterochromatin assembly and inheritance of H3K9 methylation during S phase (26).

In a recent study, heterochromatin was shown to be required for de novo assembly of CENP-A chromatin on minichromosomes containing centromeric DNA (27). However, it appears to be dispensable for the maintenance of CENP-A^{cnp1} chromatin integrity at endogenous centromeres because the distribution of centromeric CENP-A^{cnp1} remains undisturbed in *clr4* and RNAi mutants (27).

In this study, we show that the Dos1–Dos2–Cdc20 complex, previously characterized as a silencing complex, is also essential for the deposition of CENP-A^{cnp1} at centromeres during S phase.

Author contributions: M.G. and F.L. designed research; M.G., H.H., S.S., C.L., and F.L. performed research; M.G., H.H., S.S., C.L., and F.L. analyzed data; and M.G. and F.L. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

[1]To whom correspondence should be addressed. E-mail: fl43@nyu.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1214874110/-/DCSupplemental.

www.pnas.org/cgi/doi/10.1073/pnas.1214874110

These findings established a mechanistic link between DNA replication and CENP-A assembly machinery and suggest a possible mechanism for faithful inheritance of preexisting CENP-A during S phase.

Results

Dos1 and Dos2 Are Required for Proper Deposition of CENP-A at Centromeres. To investigate how Dos1 and Dos2 influence the deposition of CENP-A^{cnp1} at centromeres, we independently crossed a strain expressing CENP-A^{cnp1}–GFP into either a *dos1*Δ or *dos2*Δ mutant. In wild-type mitotic cells, centromeres cluster at the spindle pole body (SPB) on the nuclear periphery and form a single CENP-A^{cnp1}–GFP spot (Fig. 1*A*). In contrast, 21 and 18% of *dos1* and *dos2* mutant cells analyzed, respectively, show dissociation of CENP-A^{cnp1}–GFP from centromeres. More in-depth analysis of *dos1*Δ and *dos2*Δ mutants revealed that the CENP-A^{cnp1}–GFP signal not only dissociates from centromeres but often ends up as multiple distinct foci throughout the nucleus, suggesting mislocalization of CENP-A^{cnp1} to noncentromeric sites (Fig. 1*A*). Interestingly, in *dos1* and *dos2* mutants in which a single CENP-A^{cnp1}–GFP spot was observed, the fluorescent spot appeared elongated with an average length twofold longer than that observed in wild-type cells (Fig. 1*B*). This suggests that CENP-A may be spreading beyond centromeric core regions in the mutants. In fission yeast, the Dicer ribonuclease, encoded by the *dcr1*$^+$ gene, is important for heterochromatin assembly (18). Notably, despite the heterochromatin defects associated with the *dcr1*Δ mutant, none of the *dcr1*Δ cells that we analyzed showed dissociation of CENP-A^{cnp1} from centromeric cores or formed the multiple fluorescent foci typical of the *dos* mutants. In addition, the size of the CENP-A^{cnp1}–GFP single focus remained the same as that of wild type (Fig. S1). This is consistent with previous reports indicating that heterochromatin is dispensable for the maintenance of CENP-A^{cnp1} at endogenous centromeres (27).

To further characterize the pattern of CENP-A–GFP distribution at centromeric cores in the *dos1*Δ and *dos2*Δ mutants, we performed chromatin immunoprecipitation (ChIP) using an anti-GFP antibody. Consistent with our cytological observations, our ChIP results indicate that, in the *dos1* and *dos2* mutants, CENP-A^{cnp1}–GFP levels at centromeric cores are significantly reduced (Fig. 1 *C* and *D*). To investigate whether CENP-A^{cnp1} spreads beyond the core region in the *dos1*Δ and *dos2*Δ mutants, we examined the levels of CENP-A^{cnp1}–GFP at pericentromeric outer repeats (*otr*) using ChIP. In wild-type cells, *otr* regions are heterochromatic and contain very low levels of CENP-A^{cnp1}. We found that in *dos1* and *dos2* mutants, CENP-A^{cnp1}–GFP levels increase significantly at *otr* regions relative to wild-type cells, in which CENP-A^{cnp1}–GFP at these regions is undetectable (Fig. 1 *C* and *E*). These results are in agreement with the elongated CENP-A^{cnp1}–GFP signal observed in

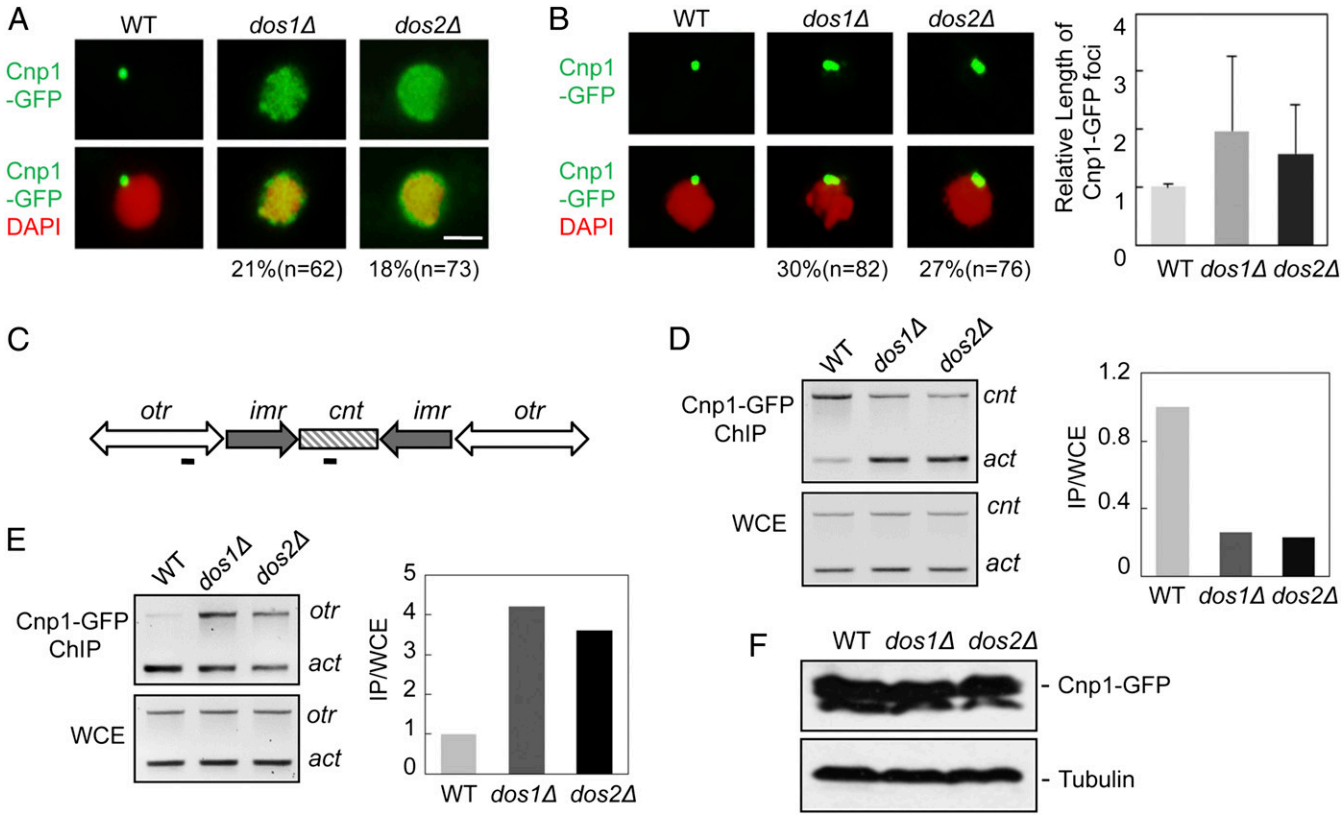

Fig. 1. Dos1 and Dos2 are required for centromeric localization of CENP-A. (*A*) In mitotic wild-type (WT) cells, CENP-A^{cnp1}-GFP (green) forms a single GFP focus due to clustering of all three centromeres. CENP-A^{cnp1}–GFP is delocalized from centromeres in *dos1*Δ and *dos2*Δ mutants. Values to the bottom indicate the proportion of mutant cells displaying CENP-A^{cnp1}–GFP delocalization. DNA was visualized by DAPI staining (red). (Scale bar, 2 μm.) (*B*) Quantification of the relative length of CENP-A^{cnp1}–GFP foci in the *dos1*Δ and *dos2*Δ mutants containing a single fluorescent spot. Values to the bottom indicate the proportion of mutant cells exhibiting CENP-A^{cnp1}–GFP foci that are ~50% longer than these in WT. (*C*) Schematic representation of a *S. pombe* centromere, which contains a central core domain (*cnt*) flanked by the *imr* and *otr* inverted repeats. Locations of primers used in ChIP assays are indicated. (*D*) CENP-A^{cnp1}–GFP is markedly reduced at centromeric cores (*cnt*) in *dos1*Δ and *dos2*Δ mutants. ChIP assays were performed with an antibody against GFP. Immunoprecipitated DNA was analyzed by competitive PCR with primers specific for a *cnt* region and a control gene, *act1*$^+$ (*act*). The relative fold enrichment shown (*Right*) was calculated by comparing the ratios of CENP-A^{cnp1}–GFP signals against control signals between ChIP and WCE fractions. The value for WT was normalized to 1.0. WCE, whole-cell extracts. (*E*) CENP-A^{cnp1}–GFP is enriched at pericentromeric *otr* regions in *dos1*Δ and *dos2*Δ mutants. An antibody against GFP was used for the ChIP. Primer pairs specific for pericentromeric *otr* regions and *act1*$^+$ were used in competitive PCR. (*F*) Western blot analysis shows that cellular CENP-A^{cnp1}–GFP levels are not affected in the *dos1*Δ and *dos2*Δ mutants. Whole-cell extracts were immunoblotted with antibodies against GFP or tubulin.

these mutants (Fig. 1*B*). Together, our observations suggest that Dos1 and Dos2 are important for preventing mislocalization of CENP-A to noncentromeric regions.

To examine whether deleting Dos1 and Dos2 can lead to changes in cellular CENP-A^{cnp1} levels, we performed Western blotting using an antibody specific for GFP. We found that cellular CENP-A^{cnp1} levels are not affected in either the *dos1*Δ or *dos2*Δ mutants (Fig. 1*F*).

Dos1 and Dos2 Are Important for Kinetochore Integrity and Centromere Function. To determine whether CENP-A^{cnp1}-GFP delocalization in the *dos1*Δ and *dos2*Δ mutants leads to defects in kinetochore assembly, we examined the distribution patterns of a GFP-tagged kinetochore protein, Cnp3/CENPC. Cnp3 is a highly conserved kinetochore protein, and its localization to centromeres depends on CENP-A^{cnp1} (28). The association of Cnp3-GFP with centromeric cores was analyzed by ChIP. Cnp3 levels were markedly reduced in *dos1*Δ and *dos2*Δ mutants (Fig. 2*A*), indicating that Cnp3 dissociates from centromeric cores in these mutants.

We reasoned that the organization of bipolar spindles would be affected in the *dos1*Δ and *dos2*Δ mutants due to defective kinetochores. To investigate this possibility, we examined bipolar spindle organization in *dos1*Δ and *dos2*Δ mutants expressing GFP-labeled α-tubulin. In wild-type *S. pombe* mitotic cells, homologous chromosomes are pulled to opposite poles of the nucleus by a straight intranuclear microtubule spindle extending between two oppositely positioned SPBs (9). In contrast, 11 and 13% of *dos1*Δ and *dos2*Δ cells at metaphase, respectively, exhibit V-shaped or astral spindles, indicative of defects in the organization of the bipolar spindle (Fig. S2). In addition, 17 and 14% of *dos1*Δ and *dos2*Δ cells, respectively, failed to segregate their chromosomes equally (Fig. S2). This is consistent with previous observations that *dos1* and *dos2* mutants exhibit lagging chromosomes and are hypersensitive to the microtubule-destabilizing drug thiabendazole (TBZ) (20, 23).

In fission yeast, histones at centromeric cores exist in a hypoacetylated state. This contrasts with mutants of CENP-A^{cnp1} loading factors, such as Mis16 and Mis18, in which centromeric histones become hyperacetylated (29). To investigate whether Dos1 and Dos2 affect the acetylation state of histones in centromeric cores, we used ChIP to examine acetylation levels in *dos1* and *dos2* mutants. We interrogated centromeric core *cnt* regions using an antibody against acetylated histone H4. We found that in both mutants the levels of acetylated H4 in *cnt* regions were significantly increased (Fig. 2*B*). These observations support the view that, like Mis16 and Mis18, Dos1 and Dos2 play a critical role in maintaining the low levels of acetylation typical of centromeric cores.

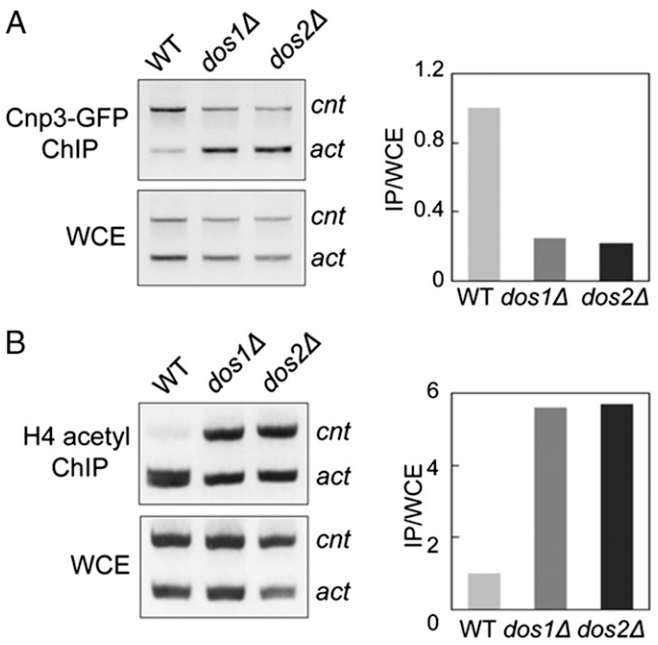

Fig. 2. Dos1 and Dos2 are essential for core centromere function. (*A*) Centromeric localization of the kinetochore protein, Cnp3–GFP, in *dos1*Δ and *dos2*Δ cells is disrupted. ChIP was performed using an anti-GFP antibody and primers specific for a *cnt* region. (*B*) *dos1*Δ and *dos2*Δ mutants show significantly higher H4 acetylation relative to wild type at centromeric cores. ChIP assays were performed with an antibody against H4 acetylation. Primer pairs specific for *cnt* regions were used.

Genetic Interactions Between Dos Proteins with Mis6 and Ams2. To examine potential functional interactions between Dos1 and Dos2 with the known CENP-A^{cnp1} loading factors, Mis6 and Ams2, we created the double mutants *dos1*Δ *mis6-302*, *dos1*Δ *ams2*Δ, *dos2*Δ *mis6-302*, and *dos2*Δ *ams2*Δ. All mutants were analyzed for growth at 24 and 34 °C. *mis6-302* is a temperature-sensitive mutant that grows slowly at 34 °C but fails to grow at 36 °C. We found that the double mutants *dos1*Δ *mis6-302* and *dos2*Δ *mis6-302* exhibit synthetic lethality at 34 °C. In contrast, no detectable growth defects could be observed in the double mutants *dos1*Δ *ams2*Δ and *dos2*Δ *ams2*Δ at either temperature (Fig. 3*A*).

Dos1–Dos2 Complex Interacts with Ams2. A plausible deduction from our genetic interaction studies is that Dos1 and Dos2 may function in the Ams2-dependent pathway for CENP-A loading. Consistent with this, both Dos1 and Dos2, as well as Ams2, are dispensable for viability, whereas deletion of Mis6 results in cell death.

To investigate whether the Dos1–Dos2 complex functions in the same pathway as Ams2, we tested for potential physical interaction between the Dos proteins and Ams2 by coimmunoprecipitation. We found that Dos1 coimmunoprecipitates with Ams2, indicating that Dos1 physically associates with Ams2 (Fig. 3*B*).

Dos1 Is Required for the Association of Ams2 with Centromeric Cores. Previous studies indicate that Ams2 associates with centromeric cores specifically during S phase (17). We therefore asked whether Dos1 is required for the localization of Ams2 to centromeric cores. *dos1*Δ cells expressing GFP-tagged Ams2 were arrested at S phase by hydroxyurea and examined for any defects in the association of Ams2 with core regions during S phase using ChIP. We found that the association of Ams2 with centromeric cores during S phase is significantly decreased in *dos1*Δ cells (Fig. 3*C*). These results suggest that Dos1 is required for the recruitment of Ams2 to centromeric cores. In wild-type cells, Mis6-GFP localizes to centromeric cores and appears as a single fluorescent spot due to centromere clustering (17). We observed that in *dos1*Δ and *dos2*Δ cells the distribution pattern of Mis6-GFP is not affected (Fig. S3), indicating that Dos1 and Dos2 are not required for the recruitment of Mis6 to centromeric cores.

Cdc20 Is Required for Deposition of CENP-A at Centromeres. We recently discovered that Dos1 and Dos2 interact with the DNA polymerase epsilon subunit, Cdc20, to facilitate inheritance of H3K9 methylation in heterochromatin during S phase (26). To investigate whether Cdc20 is also required for inheritance of CENP-A at centromeres, we used the temperature-sensitive mutant *cdc20-p7* expressing GFP-labeled CENP-A^{cnp1}. This mutant grows well at room temperature and fails to grow at 37 °C (30). It can, however, grow at 34 °C, although poorly (26). We examined the distribution pattern of CENP-A^{cnp1}–GFP in *cdc20-p7* cells at 22, 34, and 37 °C. As shown in Fig. 4*A*, at 22 °C a single nuclear CENP-A^{cnp1}–GFP spot, typical of wild-type cells, was visible for most cells. In contrast, at 34 °C, 51% of *cdc20-p7* cells analyzed showed CENP-A^{cnp1}–GFP dissociation from centromeres (Fig. 4*A*). For *cdc20-p7* cells exhibiting a single GFP spot at 34 °C, the CENP-A–GFP spot often appeared elongated relative to spots in *cdc20-p7* cells grown at 22 °C (Fig. 4*B*), suggesting that CENP-A mislocalizes to pericentromeric regions at this temperature. CENP-A^{cnp1}–GFP localization defects worsen at 37 °C: Following 8 h of incubation at this temperature,

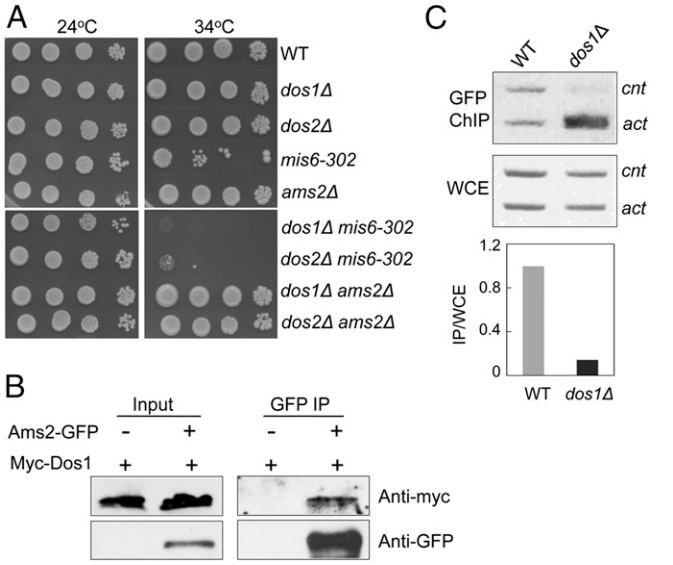

Fig. 3. Dos1 and Dos2 are required for the localization of Ams2 to centromeres. (*A*) Synthetic genetic interactions for *dos1*Δ or *dos2*Δ with *ams2*Δ or *mis6-302* mutants. Tenfold serial dilutions of cells in YES media were grown at 24 or 34 °C for 3 d. (*B*) Physical interaction between Ams2 and Dos1. Cell lysates from cells expressing Ams2–GFP and myc-Dos1 and from control cells expressing myc-Dos1 only were subjected to immunoprecipitation with an anti-GFP antibody. Precipitated proteins were analyzed by Western blotting using anti-myc or anti-GFP antibody as indicated. (*C*) Ams2's association with centromeric cores at S phase is disrupted in the *dos1*Δ mutant. ChIP assays were performed using an anti-GFP antibody for cells expressing Ams2–GFP and synchronized to S phase by hydroxyurea. Primer pairs specific for a *cnt* region were used.

more than 90% of *cdc20-p7* mutant cells display CENP-A^{cnp1}–GFP delocalization from centromeric cores. In contrast, CENP-A^{cnp1}–GFP remained as a single spot in wild-type cells incubated at all three temperatures (Fig. S4). Consistent with these observations, our ChIP analysis showed a close to 70% reduction in CENP-A^{cnp1} levels at centromeric cores in *cdc20-p7* cells at 34 °C (Fig. 4*C*). Additionally, CENP-A^{cnp1}–GFP is enriched at *otr* regions at 34 °C, compared with *cdc20-p7* cells incubated at 22 °C (Fig. 4*D*). Combined, these observations suggest a role for Cdc20 in the deposition of CENP-A at centromeric cores. We next examined whether disrupting *cdc20* results in changes in total CENP-A^{cnp1} levels. Western blot analysis showed that in *cdc20-p7* cells CENP-A^{cnp1}–GFP levels remained as in wild type at both 22 and 34 °C (Fig. S5).

Cdc20 Is Essential for Centromere Function. To examine whether disrupting *cdc20* results in chromosome-segregation defects, serial dilutions of the *cdc20-p7* cells were plated on rich medium containing TBZ and incubated at both 22 and 34 °C. We found that at 34 °C the cells display strong sensitivity to TBZ (Fig. 5*A*), suggesting that chromosome segregation may be compromised at this temperature. To further test for chromosome segregation defects in the mutant we investigated spindle organization using GFP-labeled α-tubulin. As shown in Fig. 5*B*, 12% of *cdc20-p7* mitotic cells analyzed display V-shaped or astral spindles at 34 °C relative to *cdc20-p7* mitotic cells incubated at 22 °C, in which mitotic spindles appeared normal (Fig. 5*B*). Additionally, a DAPI stain shows that in 19% of *cdc20-p7* cells, chromosomes also fail to segregate equally at 34 °C relative to mutant cells at 22 °C in which chromosome segregation proceeds normally (Fig. 5*B*).

Association of Dos2 with Centromeric Cores During S Phase Is Disrupted in a *cdc20* Mutant. We previously showed that in wild-type cells Dos2 is enriched at pericentric heterochromatin during S phase (26). In this study, we asked whether Dos2 also preferentially associates with centromeric cores during S phase. Wild-type cells expressing Dos2–TAP at endogenous levels were synchronized using hydroxyurea. Cells were then released from the S-phase block, and the association of Dos2 with centromeric cores at various stages of the cell cycle was examined using ChIP. As shown in Fig. S6, the level of Dos2 within centromeric cores increased markedly during S phase, indicating that, like Ams2 (17), Dos2 also specifically associates with these regions during S phase. Our previous study indicated that Dos2's association with pericentromeric heterochromatin requires Cdc20 (26). To investigate how Cdc20 may influence the localization of Dos2 at centromeric cores, we performed ChIP in the *cdc20-p7* mutant at both 22 and 34 °C degrees. We found that at 22 °C the association of Dos2 with core regions during S phase remained largely unchanged in *cdc20-p7* cells; in contrast, the level of Dos2 at these sites was markedly reduced during S phase in the mutant incubated at 34 °C (Fig. 5*C*). Combined, our observations indicate that the localization of Dos2 to centromeric cores during S phase depends on Cdc20.

Noncoding RNAs from Centromeric Cores Are Highly Accumulated in *dos1*Δ and *cdc20-p7*. A recent study in fission yeast shows that centromeric cores, where CENP-A^{cnp1} resides, undergo transcription by RNA Pol II, albeit at low levels. However, cryptic transcription from the centromeric cores is prevalent in mutants

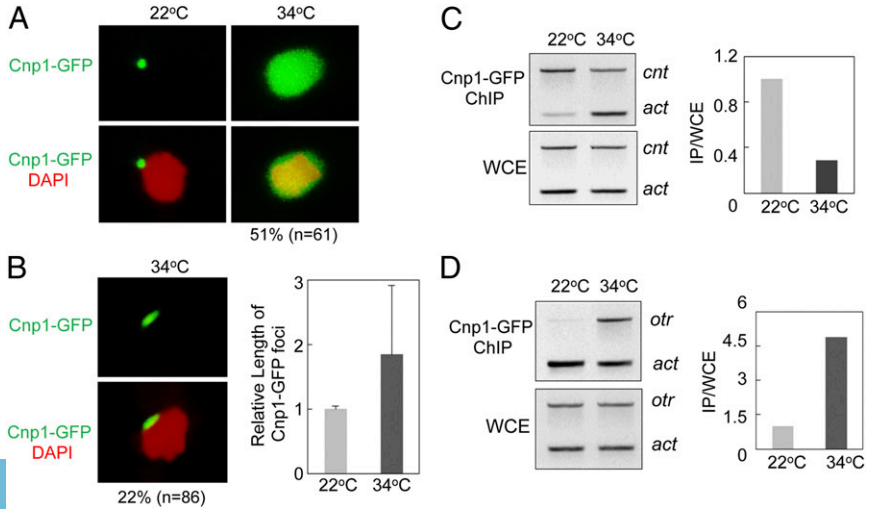

Fig. 4. Deposition of CENP-A^{cnp1}–GFP at centromeres depends on Cdc20. (*A*) CENP-A^{cnp1} –GFP delocalizes from centromeres in 51% of *cdc20-p7* mutant cells incubated at 34 °C for 8 h. In contrast, a single nuclear CENP-A^{cnp1}–GFP spot, typical of wild-type cells, was visible for most mutant cells at 22 °C. (*B*) Quantification of the relative length of CENP-A^{cnp1}–GFP foci in *cdc20-p7* cells at 34 °C with a single fluorescent focus. Values to the bottom indicate the proportion of mutant cells at 34 °C exhibiting CENP-A^{cnp1}–GFP foci 50% longer than those present in the mutant at 22 °C. (*C*) CENP-A^{cnp1}–GFP is significantly reduced in *cnt* regions in *cdc20-p7* cells at 34 °C. Wild-type and *cdc20-p7* cells were grown at 22 °C and then shifted to 34 °C for 8 h in preparation for ChIP analysis. (*D*) CENP-A^{cnp1}–GFP levels at the *otr* regions increase in *cdc20-p7* cells at 34 °C.

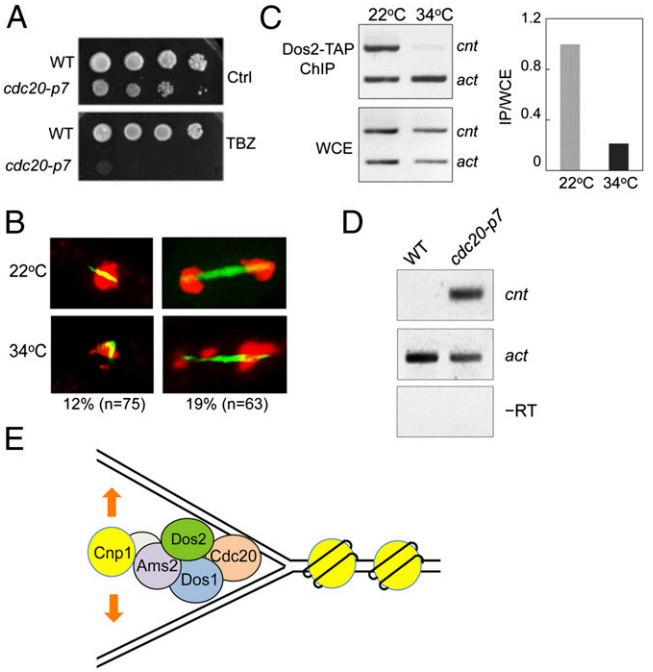

Fig. 5. Centromere function is disrupted in the *cdc20-p7* mutant. (*A*) *cdc20-p7* cells are highly sensitive to thiabendazole (TBZ) at 34 °C. Serial dilutions of indicated cultures were plated onto YES medium (*Upper*) or YES medium supplemented with 10 μg/mL TBZ (*Lower*). (*B*) Spindle organization and chromosome segregation are defective in *cdc20-p7* cells at 34 °C (*Lower*) but appear normal in most mutant cells at 22 °C (*Upper*). Values to the bottom indicate the proportion of cells displaying spindle disorganization defects (*Left*), or unequal chromosome segregation (*Right*). Spindle microtubules and DNA were visualized by GFP-tubulin (green) and DAPI (red), respectively. (*C*) Dos2 dissociates from centromeric cores at S phase in *cdc20-p7* mutant at 34 °C. ChIP assays were performed using Dos2–TAP-expressing cells synchronized to S phase. (*D*) RT-PCR analysis of core centromeric transcripts in the *cdc20-p7* mutant at 34 °C, relative to wild type at 34 °C. (*E*) Model for the loading of CENP-A at centromeres during S phase: As the DNA Pol epsilon subunit, Cdc20, catalyzes daughter-strand synthesis at centromeric cores, it also serves to recruit the Dos1–Dos2 complex. The Dos1–Dos2 complex, in turn, interacts with Ams2 directly or indirectly to facilitate the loading of CENP-A at centromeres during S phase.

defective in formation of subkinetochore chromatin, such as *cnp1* and *mis12* (31). To examine how noncoding RNA transcription from *cnt* regions is affected in the *dos1*Δ mutant, we analyzed *dos1*Δ cells by RT-PCR using primers specific for core regions. As shown in Fig. S7, centromeric transcripts are highly accumulated in *dos1* mutant cells, suggesting that Dos1 is required for regulation of these RNA transcripts. We also found that noncoding RNAs from core regions are also highly accumulated in *cdc20-p7 cells* at 34 °C, relative to wild type (Fig. 5*D*), indicating that Cdc20 may also play a role in regulating these noncoding transcripts.

Discussion

Here we demonstrate that the Dos1–Dos2–Cdc20 complex is important for assembly of CENP-A into centromeric chromatin. Disruption of each of its components results in chromosome segregation defects and mislocalization of CENP-A to noncentromeric sites. We further show that Dos1 interacts with the CENP-A assembly factor, Ams2, and is required for the recruitment of Ams2 to centromeric cores during S phase. In addition, the association of Dos2 with centromeric core regions at S phase depends on the DNA polymerase epsilon subunit, Cdc20. Our findings uncover a physical link between CENP-A deposition and DNA replication and shed light on the mechanisms involved in CENP-A deposition at centromeres during S phase. In addition, our studies further emphasize the role of DNA replication components in inheritance of epigenetic information. Based on our findings, we propose the following model: During S phase of the cell cycle, as DNA polymerase epsilon subunit, Cdc20, catalyzes daughter-strand synthesis at centromeric replication forks, it also serves to recruit the Dos1–Dos2 complex; the Dos1–Dos2 complex, in turn, promotes inheritance of CENP-A at centromeric cores through its ability to interact directly or indirectly with the CENP-A loading factor, Ams2 (Fig. 5*E*).

Precise spatial and temporal regulation of CENP-A deposition at centromeres is essential for chromosome segregation (1). CENP-A chromatin assembly is tightly coupled with cell-cycle regulation (2). In *Drosophila* and humans, preexisting CENP-A is distributed evenly among daughter centromeres following DNA replication in S phase, whereas newly synthesized CENP-A is deposited at later stages of the cell cycle (2, 6). A recent quantitative single-molecule study revealed that the number of CENP-A^{cnp1} molecules in fission yeast centromeres increases during G2 and is maintained at the same level throughout S phase, suggesting that the propagation of preexisting CENP-A^{cnp1} in fission yeast also takes place during S phase (32). The deposition of newly synthesized CENP-A depends on HJURP/Scm3 components (7–10). How parental CENP-A is reassembled into nucleosomes of daughter centromeres during S phase remains elusive. Our studies raise the intriguing possibility that DNA replication components and CENP-A assembly machinery cooperate to facilitate the reassembly of parental CENP-A into newly replicated centromeres, which may provide a potential mechanistic explanation for how inheritance of preexisting CENP-A is achieved during S phase. We speculate that in fission yeast, Cdc20 at replication forks may act in concert with Dos1, Dos2, and the S-phase–dependent CENP-A assembly factor, Ams2, to assist in the transmission of preexisting CENP-A from mother to daughter centromeres. In the future, it would be interesting to investigate how exactly the Dos1–Dos2–Cdc20 complex may contribute to the inheritance of preexisting CENP-A during centromere replication.

Although RNAi-dependent heterochromatin is required for de novo assembly of CENP-A at naïve centromeric DNA, disruption of heterochromatin has little effect on inheritance of CENP-A at endogenous loci (27) (Fig. S1). In contrast, we show that perturbation of Dos1, Dos2, and Cdc20 results in severe dissociation of CENP-A from centromeres and mislocalization of the protein to noncentromeric sites. Our data support the notion that the Dos1–Dos2–Cdc20 complex plays a direct role in the deposition of CENP-A at centromeres during S phase through interacting with Ams2. We previously demonstrated that the Dos1–Dos2–Cdc20 complex mediates inheritance of heterochromatin silencing and H3K9 methylation at pericentromeric regions (26). Thus, the complex is pivotal for faithful copying of epigenetic information at both centromeric and pericentromeric regions. This dual function of the Dos1–Dos2–Cdc20 complex may be explained based on its ability to associate with different protein partners at the two distinct chromatin environments. A previous study has shown that the DNA polymerase alpha subunit, Mcl1, is required for incorporation of CENP-A at centromeric cores and for heterochromatin silencing at pericentromeric regions (33). It is conceivable that Mcl1 may functions together with Dos1–Dos2–Cdc20 to mediate the inheritance of CENP-A at centromeric cores and pericentromeric silencing.

Core centromeric RNA transcripts have been identified in a variety of organisms (34, 35). Recently, it was shown that core centromeric regions in fission yeast, where CENP-A^{cnp1} resides, are also transcribed, albeit at low levels. Interestingly, in mutants defective in kinetochore integrity, including *cnp1* and *mis12*, abundant centromeric RNAs were identified (31). These transcripts are transcribed from both strands by RNA Pol II but are distinct from those transcribed from pericentromere repeats (31). We found that in *dos1* and *cdc20* mutants core centromeric transcripts are also highly accumulated. Little is known about how transcripts from centromeric cores may influence CENP-A deposition (31). Our studies implicate the Dos1–Dos2–Cdc20 complex in the regulation of noncoding RNAs transcripts from centromeric cores.

Materials and Methods

Strains, Media, and Genetic Analysis. Fission yeast strains used in this study are listed in Table S1. Growth media and genetic methods for *S. pombe* were performed as described (36). Cells synchronization was performed by treatment with hydroxyurea (26).

Immunoprecipitation. Cells synchronized to S phase with hydroxyurea were collected and lysed in Homogenization buffer (HB; 25 mM MOPS, pH 7.2, 15 mM $MgCl_2$, 15 mM EGTA, 1 mM DTT, 1% Triton X-100 with phosphatase inhibitor and protease inhibitors). Immunoprecipitation was conducted as described (36). Lysates were precleared with protein A agarose beads and incubated with anti-GFP antibody (ab290; Abcam) at 4 °C for 2 h. After washing with HB buffer three times, proteins were eluted in SDS buffer. Eluates were analyzed by Western blotting using commercial anti-myc (C3956; Sigma), anti-GFP (ab290; Abcam), and α-tubulin (ab6160; Abcam) antibodies.

ChIP. ChIP assays were carried out as described (21). Briefly, cells were grown to log phase at 22 °C and cross-linked by treating with 1% formaldehyde. For the *cdc20-p7* mutant strain, cells were grown at 22 °C and then shifted to 34 °C for 8 h before cross-linking. Immunoprecipitation was performed with protein S agarose (Novagen) and either anti-GFP or anti-acetyl H4 antibody (06-866; Upstate). Precipitated DNA was suspended in 30 μL of Tris-EDTA (TE) buffer. Two microliters of ChIP or WCE samples were analyzed by competitive PCR using oligonucleotides specific to centromeric core regions and to the control gene, *act1*$^+$. To ensure that PCR products lay within the linear range of amplification, PCRs were carried out with serially diluted templates. For each PCR 27–30 amplification cycles were used. PCR products were separated on a 1.7% (weight/vol) agarose gel and poststained with ethidium bromide. Primers used are listed in Table S2. All experiments were conducted in triplicate. Quantitations were performed using ImageJ 1.46r software.

RT-PCR. Total RNA from actively diving cells grown at 22 °C was extracted using TRizol (Invitrogen). After treatment of the purified RNA with DNase I (Promega), 50 ng of RNA were analyzed by RT-PCR in a 25-μL reaction volume using a One-Step RT-PCR kit (Qiagen). Equal loading of RNA samples was assessed by amplification of the internal control gene, *act1*$^+$. Primers used for RT-PCR are listed in Table S2.

Microscopy. Fluorescent images were taken with a Delta Vision System (Applied Precision). SoftWoRX2.50 (Applied Precision) was used for processing the final projections.

The standard procedure for staining and analysis of cells using DAPI was used.

ACKNOWLEDGMENTS. We thank Paul Nurse, Yoshinori Watanabe, Mitsuhiro Yanagida, and the Yeast Genetic Resource Center of Japan for kindly providing strains used in this study and W. Zacheus Cande for critical reading of the manuscript. This study was supported by a startup fund from New York University (to F.L.).

1. Morris CA, Moazed D (2007) Centromere assembly and propagation. *Cell* 128(4): 647–650.
2. Black BE, Cleveland DW (2011) Epigenetic centromere propagation and the nature of CENP-a nucleosomes. *Cell* 144(4):471–479.
3. Blower MD, Karpen GH (2001) The role of Drosophila CID in kinetochore formation, cell-cycle progression and heterochromatin interactions. *Nat Cell Biol* 3(8):730–739.
4. Probst AV, Dunleavy E, Almouzni G (2009) Epigenetic inheritance during the cell cycle. *Nat Rev Mol Cell Biol* 10(3):192–206.
5. Schuh M, Lehner CF, Heidmann S (2007) Incorporation of Drosophila CID/CENP-A and CENP-C into centromeres during early embryonic anaphase. *Curr Biol* 17(3):237–243.
6. Jansen LE, Black BE, Foltz DR, Cleveland DW (2007) Propagation of centromeric chromatin requires exit from mitosis. *J Cell Biol* 176(6):795–805.
7. Foltz DR, et al. (2009) Centromere-specific assembly of CENP-a nucleosomes is mediated by HJURP. *Cell* 137(3):472–484.
8. Dunleavy EM, et al. (2009) HJURP is a cell-cycle-dependent maintenance and deposition factor of CENP-A at centromeres. *Cell* 137(3):485–497.
9. Pidoux AL, et al. (2009) Fission yeast Scm3: A CENP-A receptor required for integrity of subkinetochore chromatin. *Mol Cell* 33(3):299–311.
10. Camahort R, et al. (2007) Scm3 is essential to recruit the histone h3 variant cse4 to centromeres and to maintain a functional kinetochore. *Mol Cell* 26(6):853–865.
11. Carroll CW, Straight AF (2006) Centromere formation: From epigenetics to self-assembly. *Trends Cell Biol* 16(2):70–78.
12. Verdaasdonk JS, Bloom K (2011) Centromeres: Unique chromatin structures that drive chromosome segregation. *Nat Rev Mol Cell Biol* 12(5):320–332.
13. Takahashi K, Takayama Y, Masuda F, Kobayashi Y, 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(1455):595–606, discussion 606–607.
14. Takayama Y, et al. (2008) Biphasic incorporation of centromeric histone CENP-A in fission yeast. *Mol Biol Cell* 19(2):682–690.
15. Takahashi K, Chen ES, Yanagida M (2000) Requirement of Mis6 centromere connector for localizing a CENP-A-like protein in fission yeast. *Science* 288(5474):2215–2219.
16. Okada M, et al. (2006) The CENP-H-I complex is required for the efficient incorporation of newly synthesized CENP-A into centromeres. *Nat Cell Biol* 8(5): 446–457.
17. Chen ES, Saitoh S, Yanagida M, Takahashi K (2003) A cell cycle-regulated GATA factor promotes centromeric localization of CENP-A in fission yeast. *Mol Cell* 11(1):175–187.
18. Kloc A, Zaratiegui M, Nora E, Martienssen R (2008) RNA interference guides histone modification during the S phase of chromosomal replication. *Curr Biol* 18(7):490–495.
19. Chen ES, et al. (2008) Cell cycle control of centromeric repeat transcription and heterochromatin assembly. *Nature* 451(7179):734–737.
20. Li F, et al. (2005) Two novel proteins, dos1 and dos2, interact with rik1 to regulate heterochromatic RNA interference and histone modification. *Curr Biol* 15(16):1448–1457.
21. Li F, et al. (2008) Lid2 is required for coordinating H3K4 and H3K9 methylation of heterochromatin and euchromatin. *Cell* 135(2):272–283.
22. Nakayama J, Rice JC, Strahl BD, Allis CD, Grewal SI (2001) Role of histone H3 lysine 9 methylation in epigenetic control of heterochromatin assembly. *Science* 292(5514): 110–113.
23. Horn PJ, Bastie JN, Peterson CL (2005) A Rik1-associated, cullin-dependent E3 ubiquitin ligase is essential for heterochromatin formation. *Genes Dev* 19(14):1705–1714.
24. Hong EJ, Villén J, Gerace EL, Gygi SP, Moazed D (2005) A cullin E3 ubiquitin ligase complex associates with Rik1 and the Clr4 histone H3-K9 methyltransferase and is required for RNAi-mediated heterochromatin formation. *RNA Biol* 2(3):106–111.
25. Thon G, et al. (2005) The Clr7 and Clr8 directionality factors and the Pcu4 cullin mediate heterochromatin formation in the fission yeast Schizosaccharomyces pombe. *Genetics* 171(4):1583–1595.
26. Li F, Martienssen R, Cande WZ (2011) Coordination of DNA replication and histone modification by the Rik1-Dos2 complex. *Nature* 475(7355):244–248.
27. Folco HD, Pidoux AL, Urano T, Allshire RC (2008) Heterochromatin and RNAi are required to establish CENP-A chromatin at centromeres. *Science* 319(5859):94–97.
28. Tanaka K, Chang HL, Kagami A, Watanabe Y (2009) CENP-C functions as a scaffold for effectors with essential kinetochore functions in mitosis and meiosis. *Dev Cell* 17(3): 334–343.
29. Hayashi T, et al. (2004) Mis16 and Mis18 are required for CENP-A loading and histone deacetylation at centromeres. *Cell* 118(6):715–729.
30. D'Urso G, Nurse P (1997) Schizosaccharomyces pombe cdc20+ encodes DNA polymerase epsilon and is required for chromosomal replication but not for the S phase checkpoint. *Proc Natl Acad Sci USA* 94(23):12491–12496.
31. Choi ES, et al. (2011) Identification of noncoding transcripts from within CENP-A chromatin at fission yeast centromeres. *J Biol Chem* 286(26):23600–23607.
32. Lando D, et al. (2012) Quantitative single-molecule microscopy reveals that CENP-A (Cnp1) deposition occurs during G2 in fission yeast. *Open Biol* 2(7):120078.
33. Natsume T, et al. (2008) A DNA polymerase alpha accessory protein, Mcl1, is required for propagation of centromere structures in fission yeast. *PLoS ONE* 3(5):e2221.
34. Wong LH, et al. (2007) Centromere RNA is a key component for the assembly of nucleoproteins at the nucleolus and centromere. *Genome Res* 17(8):1146–1160.
35. Topp CN, Zhong CX, Dawe RK (2004) Centromere-encoded RNAs are integral components of the maize kinetochore. *Proc Natl Acad Sci USA* 101(45):15986–15991.
36. Moreno S, Klar A, Nurse P (1991) Molecular genetic analysis of fission yeast Schizosaccharomyces pombe. *Methods Enzymol* 194:795–823.