Condensin phosphorylated by the Aurora-B-like kinase Ark1 is continuously required until telophase in a mode distinct from Top2

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Summary

Condensin is a conserved protein complex that functions in chromosome condensation and segregation. It has not been previously unequivocally determined whether condensin is required throughout mitosis. Here, we examined whether *Schizosaccharomyces pombe* condensin continuously acts on chromosomes during mitosis and compared its role with that of DNA topoisomerase II (Top2). Using double mutants containing a temperature-sensitive allele of the condensin SMC2 subunit *cut14* (*cut14-208*) or of *top2*, together with the cold-sensitive *nda3-KM311* mutation (in β-tubulin), temperature-shift experiments were performed. These experiments allowed inactivation of condensin or Top2 at various stages throughout mitosis, even after late anaphase. The results established that mitotic chromosomes require condensin and Top2 throughout mitosis, even in telophase. We then showed that the Cnd2 subunit of condensin (also known as Barren) is the target subunit of Aurora-B-like kinase Ark1 and that Ark1-mediated phosphorylation of Cnd2 occurred throughout mitosis. The phosphorylation sites in Cnd2 were determined by mass spectrometry, and alanine and glutamate residue replacement mutant constructs for these sites were constructed. Alanine substitution mutants of Cnd2, which mimic the unphosphorylated protein, exhibited broad mitotic defects, including at telophase, and overexpression of these constructs caused a severe dominant-negative effect. By contrast, glutamate substitution mutants, which mimic the phosphorylated protein, alleviated the segregation defect in Ark1-inhibited cells. In telophase, the condensin subunits in *cut14-208* mutant accumulated in lumps that contained telomeric DNA and proteins that failed to segregate. Condensin might thus serve to keep the segregated chromosomes apart during telophase.

Key words: Condensation, Segregation, Condensin, Aurora kinase, Top2

Introduction

Mitotic chromosome condensation is an important event that is a prerequisite for accurate chromosome segregation in anaphase (Koshland and Strunnikov, 1996; McHugh and Heck, 2003; Swedlow and Hirano, 2003; Yanagida, 2005). Although mitotic chromosome condensation has been well described overall, the precise details of the relevant molecular mechanisms have remained enigmatic (Belmont, 2006; Gassmann et al., 2004). Even in unicellular eukaryotic organisms, such as yeast, interphase chromatin DNA is already compacted ~1000-fold in the nucleus, relative to the length of the naked DNA duplex. Forming properly condensed chromosomes upon entry into mitosis at prophase requires a further compaction, by severalfold, of interphase chromatin DNA (Bak et al., 1977; Earnshaw, 1988; Kireeva et al., 2004). In the fission yeast *Schizosaccharomyces pombe,* which has a small genome, chromatin condenses ~fivefold upon mitotic entry, judging from fluorescence in situ hybridization (FISH) images of chromosome arms (Saka et al., 1994). This is a marginal degree of mitotic condensation, but it is absolutely essential for proper segregation. The condensation might be necessary to promote the final compaction and resolve sister chromatids by metaphase and produce a chromosomal architecture that can endure segregation (e.g. against the pulling force of the mitotic spindle). Another possibility is that chromosome condensation serves to remove the many proteins and RNAs that bind to interphase chromosomes and that could interfere with segregation (Yanagida, 2009). These two views are not mutually exclusive.

The evolutionarily conserved condensin complex plays a fundamental role in mitotic chromosome condensation (Hirano, 2005; Hudson et al., 2009). This complex has DNA-dependent ATPase activity, which promotes positive DNA supercoiling (Kimura and Hirano, 1997). ATPase motifs are present in the two ‘structural maintenance of chromosomes’ (SMC) (Strunnikov et al., 1993) subunits (Cut3 and Cut14), which also have coiled-coil and hinge regions; three non-SMC condensin (Cnd1, Cnd2 and Cnd3) subunits are bound to the ATPase-containing globular domains of the SMC heterodimer (Yoshimura et al., 2002) (see Fig. 5A). The mitotic functions of condensin seem to be mediated by its phosphorylation in mitosis. The kinase Cdc2 activates the DNA supercoiling activity of the *Xenopus* condensin holocomplex in vitro (Kimura et al., 1998). In fission yeast, the N-terminal T19 residue of the SMC condensin subunit Cut3 (also known as SMC4) is phosphorylated by mitotic kinase Cdc2, and an unphosphorylatable mutant causes a severe defect in mitotic condensation owing to the retention of condensin in the cytoplasm (Nakazawa et al., 2008; Sutani et al., 1999). The requirement of kinase Aurora B for condensin recruitment to mitotic chromosomes was initially reported in *Drosophila* (Giet and Glover, 2001).

Journal of Cell Science

Studies in budding yeast have also shown that mitotic chromosome condensation requires a functional Aurora B kinase (Lavoie et al., 2004). Studies of vertebrate condensin suggested that Aurora B contributes to the efficient localization of condensin I (Lipp et al., 2007; Takemoto et al., 2007) and/or to the accumulation of condensin II at the kinetochore (Ono et al., 2004). In fission yeast, Cdc2 phosphorylation is not sufficient for docking condensin to mitotic chromatin, and phosphorylation by both Cdc2 and Ark1 (the fission yeast Aurora B homologue) is necessary (Nakazawa et al., 2008). An essential role of the polo kinase in ensuring anaphase-specific condensation has also been recently shown in budding yeast (St-Pierre et al., 2009).

Evidence for the essential role of condensin in mitotic chromosome condensation and subsequent segregation has been obtained by inactivating one of the condensin subunits before mitotic entry with the use of temperature-sensitive (ts) mutations, transposon insertion, conditional shut-off and antibody or RNA interference (RNAi) knockdown (Bhat et al., 1996; Hagstrom et al., 2002; Hirano and Mitchison, 1994; Hudson et al., 2003; Ono et al., 2004; Saka et al., 1994; Strunnikov et al., 1995). A study to examine the role of condensin after metaphase was performed by adding antibodies against condensin to *Xenopus* in vitro extracts immediately after the addition of Ca^{2+}, for the release from metaphase, and a chromosome non-disjunction phenotype was observed (Wignall et al., 2003). Similarly, antibody blocking of condensin was performed in frog egg extracts after condensation, and a resulting deformation of mitotic chromosomes found (Hirano and Mitchison, 1994). In vivo, the fate of the mitotic chromosome was examined after condensation in the presence of nocodazole, using budding yeast temperature-sensitive condensin mutants; after the shift to the restrictive temperature, the amount of condensed rDNA was substantially diminished, suggesting that condensin is required after condensation (Lavoie et al., 2002). Nevertheless, to our knowledge, no attempt has been made to examine the role of condensin after chromosome segregation.

In this study, we investigated the effect of condensin inactivation through two approaches. The first approach was to employ a specific double mutant that is suitable for examining the continuing role of condensin in mitosis. This mutant contains the cold-sensitive (cs) β-tubulin *nda3-KM311* mutation and the temperature-sensitive *cut14-208* mutation, and it was used for one- and two-step temperature shift-up experiments. Our second approach was to analyze the phosphorylation sites of condensin subunits during mitosis. We identified ~18 mitotic phosphorylation sites by mass spectrometry and focused on the role of sites phosphorylated by the Aurora-B-like kinase Ark1. These sites included the phosphorylated S52 residue in the non-SMC Cnd2 subunit (also known as Barren). Phosphorylation-specific antibodies directed against this residue showed that the intense mitotic phosphorylation of Cnd2 was dependent on the presence of Ark1. We constructed several alanine substitution *cnd2* mutants, whose severe defects suggested a continuous role for phosphorylated condensin during mitosis. As DNA topoisomerase II (Top2) is also required for both condensation and segregation (Uemura et al., 1987), we also examined in detail the phenotypes of the *nda3*-cs *top2*-ts double mutant and compared these phenotypes with those of the *nda3*-cs *cut14*-ts mutant. Our results strongly suggest that condensin and Top2 are both required throughout mitosis for proper chromosome maintenance, although they might influence distinct chromosomal areas.

Results

Inactivation of condensin after mitotic entry causes missegregation

We first addressed whether *S. pombe* condensin is required after chromosome condensation in mitosis. Previously, defects in condensation and segregation have been observed when *S. pombe* temperature-sensitive condensin mutants comprising largely G2 phase cells were transferred into restrictive conditions (Aono et al., 2002; Saka et al., 1994). It was thus of interest to inactivate condensin after mitotic entry to examine its immediate effect on segregation. For this purpose, the temperature-sensitive mutant *cut14-208* (Saka et al., 1994) was employed. The *cut14-208* mutant protein, which contains the mutation S861P (Sutani and Yanagida, 1997) in the coiled-coil region, remains functional at permissive temperatures (26–30°C) but is quickly inactivated by a change of temperature to 36°C (restrictive temperature). The *cut14-208* mutant was crossed with the cold-sensitive β-tubulin mutant *nda3-KM311* (Hiraoka et al., 1984) to obtain the *nda3*-cs *cut14*-ts double mutant. The Nda3-KM311 protein fails to assemble microtubules at 20–22°C but is rapidly reactivated by a shift to 30–36°C. Thus, the double-mutant strain produces colonies at 30°C but not at 22°C or 36°C (Fig. 1A).

A series of results (Fig. 1B–F) established that condensin has to be functional even after chromosome condensation. The double-mutant cells arrested after entry into mitosis at 20°C: they contained condensed chromosomes but, owing to the absence of assembled microtubules, were unable to proceed further. At 36°C (the permissive temperature for *nda3*), however, the double-mutant cells were released for mitotic progression following the reactivation of tubulin, whereas the condensin mutant protein Cut14-208 was inactive. The control single-mutant *nda3*-cs and the double-mutant *nda3*-cs *cut14*-ts strains were first grown at 30°C and then shifted to 20°C for 8 hours (Fig. 1B, $Nda3^{-}$ $Cut14^{+}$). Both strains arrested at an early mitotic stage, revealing condensed chromosomes (Fig. 1C) [DNA was stained with the fluorescent probe 4,6-diamino-2-phenylindole (DAPI)]. The cultures were then shifted to 36°C (Fig. 1B, $Nda3^{+}$ $Cut14^{-}$ for the double mutant) and aliquots were taken at intervals for 60 minutes. After 10 minutes (corresponding to early anaphase) and 15 minutes (corresponding to late anaphase), the double-mutant cells where condensin was inactivated post-condensation showed frequent abnormal segregation, whereas normal segregation was observed for the control single-mutant *nda3* strain (Fig. 1D); ~50% of the double-mutant cells displayed highly streaked or extended chromosomal DNA (Fig. 1D, arrows).

Quantitative results (Fig. 1E) showed that the percentage of early mitotic cells (designated condensed) of the control single *nda3* mutant (left-hand panel) decreased to the minimal level after 10 minutes, whereas the percentage of cells with partially separated chromosomes peaked at 40% at 5 minutes after the temperature shift. Normal chromosome segregation (two nuclei) had occurred in 60–65% of the cells at 10–15 minutes. In the double mutant with inactive condensin, the percentage of cells with partially segregated chromosomes also peaked at 40% at 5 minutes after the temperature shift, but chromosomes in less than 20% of the cells had segregated into the two daughter nuclei at 10–15 minutes (right-hand panel). The percentage of cells with the aberrant extended chromosomes instead peaked at 55% at ~10–20 minutes after the shift. The segregating daughter nuclei observed in the double mutant were frequently abnormal and differently sized, and they displayed trailing chromosomes in the middle of the cell. Thus, condensin

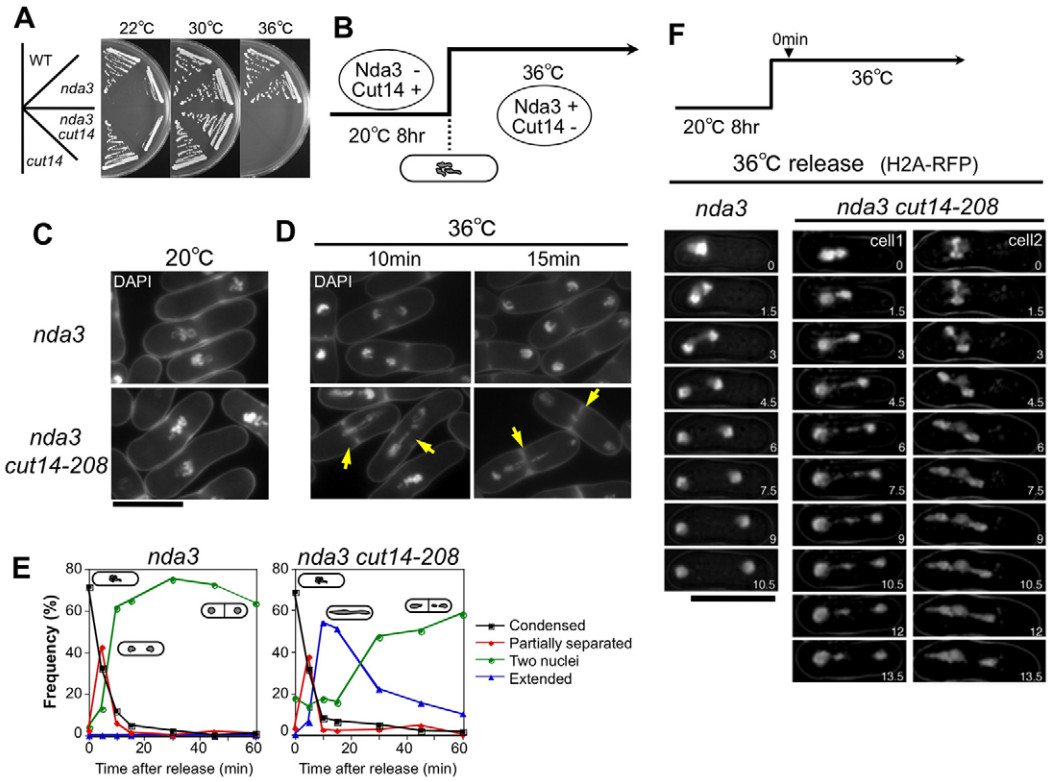

Fig. 1. Condensin Cut14 is required after condensation. (**A**) The single *nda3* and *cut14* mutants and the double mutant produce colonies at 30°C. WT, wild type. (**B**) A schematic of the temperature shift-up experiment using the double mutant. (**C**) The single *nda3* mutant and double-mutant cells arrested in mitosis stained with DAPI. (**D**) Mitotically arrested cells were released for 10 and 15 minutes by a shift to 36°C and stained with DAPI. The arrows indicate abnormally extended chromosomes. (**E**) The percentage of cells with the indicated phenotypes observed in the single *nda3* (left-hand panel) and the double-mutant *nda3 cut14* cells (right-hand panel). (**F**) Time-lapse micrographs of the temperature-shift experiment depicted at the top. See the captions of the supplementary material Movies 1 and 2 for the detailed protocol. Chromatin DNA was visualized using a gene encoding RFP-tagged histone H2A under the control of the native promoter. The numbers in the panels indicate time in minutes. Scale bars: 10 μm.

inactivation in the post-condensation period causes an aneuploidy-like situation or the physical failure in chromosome segregation.

Movies of living cells were taken with a DeltaVision microscope. The histone H2A gene (*hta1*$^{+}$) tagged with red fluorescent protein (RFP) was chromosomally integrated under its native promoter in the single *nda3*-cs and the double *nda3*-cs *cut14*-ts mutants. Time-lapse micrographs of representative cells are shown in Fig. 1F (supplementary material Movies 1 and 2). In the control *nda3* mutant cell, chromosomes had separated normally at 6–10 minutes after the release to 36°C. For the condensin-inactivated double mutant (right-hand panels), aberrant chromosomes (streaked in cell 1 and decondensed in cell 2) were observed as soon as 3 minutes after the shift. The ‘streaking’ segregation, as seen in cell 1, was more frequent than the decondensation abnormality seen in cell 2. Thus, the inactivation of Cut14 and the resulting abnormal chromosome segregation occur very rapidly, at the order of <1 minute. In cell 1, a part of the aberrant chromosomes trailed and remained in the middle of the cell by late anaphase. In cell 2, the two decondensed nuclei at the end of chromosome missegregation were large (left) and small (right), which was also observed in cell 1, suggesting that the chromosomes in these cells had unequally segregated, probably owing to the previously described kinetochore defects (Takahashi et al., 1994). Taken together, these observations indicate that condensin in *S. pombe* must be continuously active even after chromosome condensation – otherwise, chromosomes might revert to a state resembling that of unsegregated and decondensed interphase chromosomes.

To confirm that cell cycle progression resumed in mutant cells after the transfer to 36°C, GFP-tagged mitotic cyclin (Cdc13) and securin (Cut2), which are targets of ubiquitin-mediated proteolysis during the transition from metaphase to anaphase in *S. pombe* (Yanagida, 2005), were chromosomally integrated under the control of their respective native promoters, and these proteins were followed after the temperature shift. As shown in supplementary material Fig. S1A,B, the Cdc13–GFP and Cut2–GFP signals had disappeared by 15 minutes after the shift to 36°C (or after 12 minutes following incubation at 30°C for 9 minutes; see below) in the *nda3*-cs *cut14*-ts double mutant, which displayed aberrant anaphase chromosomes, and in the single *nda3* mutant, which had a normal anaphase. Thus, anaphase-promoting ubiquitin-mediated proteolytic events took place in the double-mutant cells, even though chromosome segregation was severely inhibited. We also tested whether chromosomal DNA was broken by the pulling force of the mitotic spindle during the aberrant mitosis in the double-mutant cells. Judging from the pulsed-field electrophoretic data, chromosomal DNA remained intact during the aberrant mitosis in the double mutant after the shift to 36°C for 0–33 minutes (supplementary material Fig. S1C).

Inactivation of condensin after entry into anaphase causes defects in telophase

The above experiments suggested that the requirement for *S. pombe* condensin continued after mitotic condensation. The next question that arose concerned whether condensin was required after anaphase and, if so, what would happen to chromosomes if condensin were inactivated in late anaphase. Indeed, telophase nuclei of *S. pombe* are still enriched in condensin (Nakazawa et al., 2008), suggesting that condensin has a role in late anaphase. To explore this issue, we performed two-step temperature-shift experiments (Fig. 2A). The double-mutant cells were first arrested in mitosis at 20°C, after which they were transferred to 30°C (the permissive temperature for both the *cut14* and *nda3* mutants) for an incubation of 9 minutes. During this time, the mutant cells could undergo early anaphase, and ~50% of the cells showed partially or fully separated chromosomes (Fig. 2B, middle panels). The temperature was then changed to 36°C to inactivate condensin ($Nda3^{+}$ $Cut14^{-}$). At 21 minutes, 12 minutes after the shift to 36°C, many of the double-mutant cells had undergone aberrant chromosome segregation, as judged by the frequent appearance of streaked and trailing chromosomes (Fig. 2B, arrows). By contrast, the control single-mutant cells underwent normal chromosomal segregation. Quantitative timecourse results (Fig. 2C) showed a sharp rise of aberrantly extended chromosomes in ~40% of the total double-mutant cells 12 minutes after the shift from 30°C to 36°C (21 minutes after the first shift). The control single-mutant cells did not produce such abnormally extended chromosomes.

The above results strongly suggested that condensin was required even after the bulk of the chromosomes had segregated. We next monitored this chromosome missegregation at the late segregation stages in live cells (Fig. 2D) by employing single- and double-mutant strains that expressed histone-H2A–RFP; the experimental protocol is depicted at the top of Fig. 2D (supplementary material Movies 3, 4 and 5). Cultures arrested at 20°C for 8 hours were briefly released for 7 minutes at 30°C, followed by inactivation of the temperature-sensitive Cut14 protein by a shift to 36°C. After the latter shift, we often observed late anaphase in the *nda3 cut14* double-mutant cells, in which portions of the segregating chromosomes were pulled back to the middle of the cell, a phenomenon that was not seen in the *nda3* single mutant. These portions of chromosomal DNA formed a ball-like structure (Fig. 2D, arrow in cell 1) that, unexpectedly, contained concentrated condensin (see below). This structure (designated as the 'lump' hereafter) appeared to be connected to the main bodies of the chromosomes at each end of the dividing cell, judging from the way it moved, and it was observed only at late stages of mitosis, suggesting that it was non-segregated chromosomal DNA persisting in the final stage of mitosis. Another aberrant anaphase cell type (Fig. 2D, cell 2), although less frequent than cell 1, underwent chromosome decondensation after chromosome segregation in

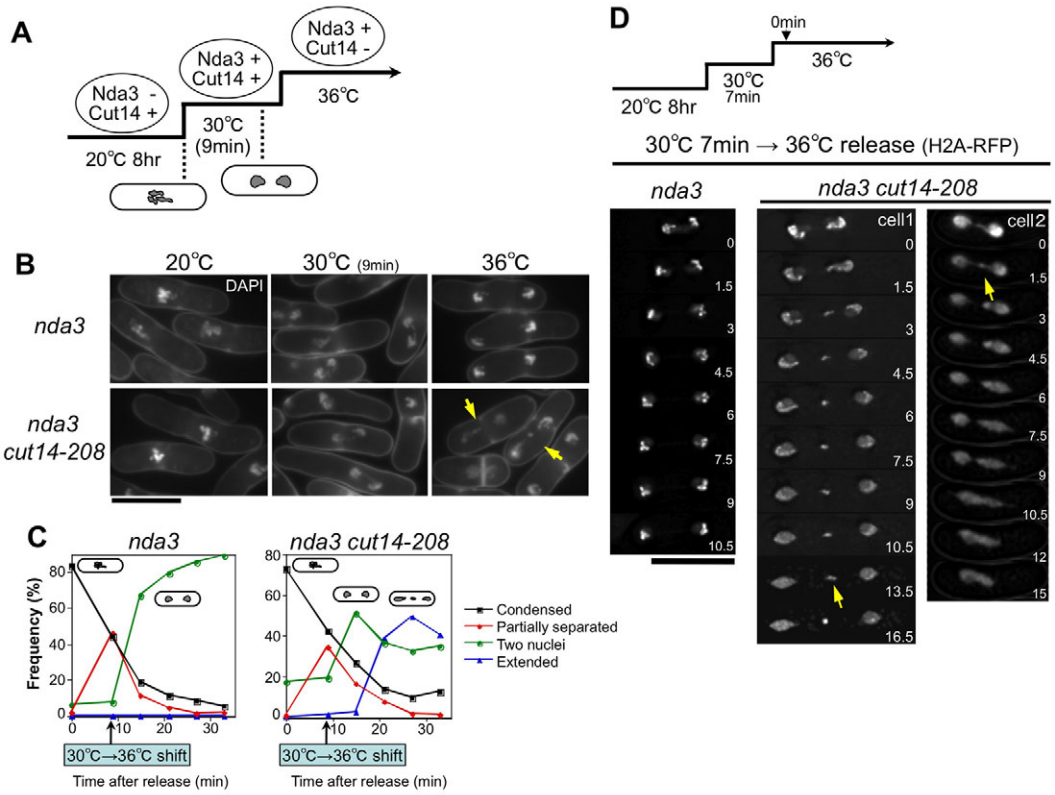

Fig. 2. Condensin Cut14 is required after anaphase. (**A**) The protocol for the two-step shift-up experiment is depicted (see the text for details). (**B**) The indicated mutants were arrested at 20°C for 8 hours (left-hand panels). The cells were released to 30°C for 9 minutes to permit spindle formation and the onset of anaphase without the inactivation of Cut14 (middle panels). Cells were shifted to 36°C to inactivate the mutant Cut14 (lower right-hand panel). In the double mutant, aberrant chromosomes were produced in late mitosis (arrows). (**C**) The percentage of cells with the indicated phenotypes observed in the single *nda3* (left-hand panel) and the double-mutant *nda3 cut14* cells (right-hand panel). (**D**) The experimental protocol of the two-step temperature shift is shown at the top. Time-lapse micrographs (the numbers indicate the time in minutes) of the single- and double-mutant cells expressing histone-H2A–RFP are shown. The arrows indicate the lump structure. Scale bar: 10 μm.

anaphase was nearly completed. The lump was also seen after 1.5 minutes in these cells, but it disappeared in late anaphase owing to decondensation. Merging of the previously segregated chromosomes also occurred. Taken together, we conclude that condensin is required for maintaining chromosome structure, even after anaphase.

Severe and distinct defects following inactivation of Top2 after anaphase

Top2 is required not only for chromosome condensation but also for segregation in *S. pombe* (Uemura et al., 1987). We attempted to compare the defects produced in the *nda3*-cs *top2-191*-ts double mutant with those in the *nda3*-cs *cut14*-ts mutant described above, by the analysis of live cells. Although *top2-191* is a standard mutant strain, Cooper and colleagues have reported that the mutant enzyme possessed the 'non-canonical' Top2-mediated activity at 19°C (Germe et al., 2009). We hence also employed another temperature-sensitive mutant strain *top2-342* for comparison with *top2-191*. Two kinds of temperature-shift experiments, one- and two-step, were performed (Fig. 3A,B, top panels). In the one-step shift-up experiment (Fig. 3A), in which Top2 was inactivated after entry into mitosis, we found double-mutant cells exhibited very severe defects, with the segregation of the bulk of whole chromosomes blocked (supplementary material Movie 6). Individual condensed chromosomes and the central nucleolar region (which had a round and smooth appearance; Fig. 3A, arrowheads) (Hirano et al., 1989; Nakazawa et al., 2008) did not segregate properly in both strains.

In the two-step shift-up experiments (Fig. 3B), cells were incubated for 10 minutes at 28°C, the permissive temperature for both the *top2* and *nda3* mutants, and then shifted to 36°C. Both of the double-mutant *top2* strains produced a central balloon-shaped

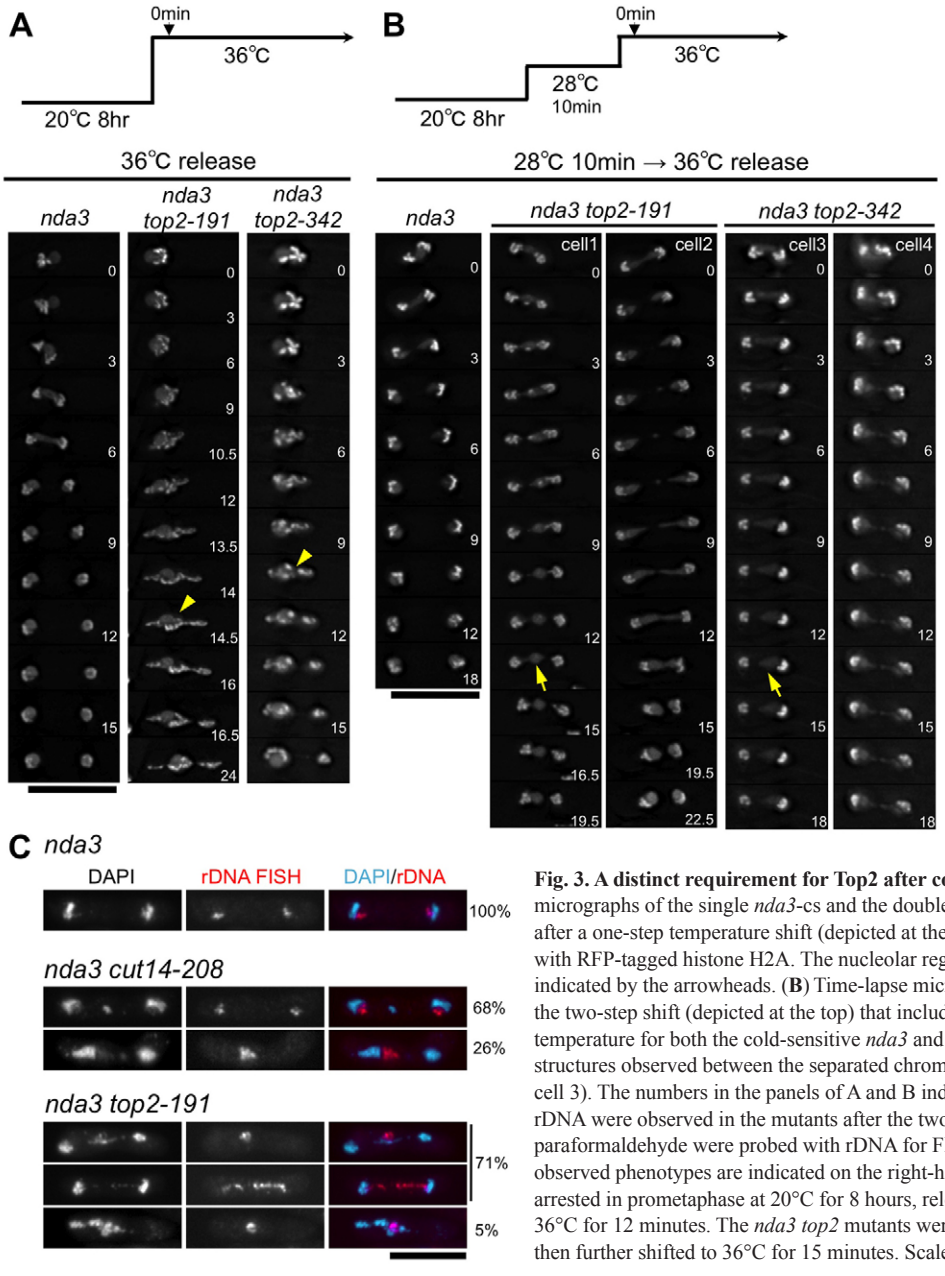

Fig. 3. A distinct requirement for Top2 after condensation and anaphase. (**A**) Time-lapse micrographs of the single *nda3*-cs and the double *nda3*-cs *top2-191*-ts or *top2-342*-ts mutant cells after a one-step temperature shift (depicted at the top) are shown. Chromosomes were visualized with RFP-tagged histone H2A. The nucleolar region, which has a round and smooth appearance, is indicated by the arrowheads. (**B**) Time-lapse micrographs of single- and double-mutant cells after the two-step shift (depicted at the top) that included the temporal transfer to 28°C (the permissive temperature for both the cold-sensitive *nda3* and the temperature-sensitive *top2*). The balloon structures observed between the separated chromosomal DNAs are indicated by arrows (cell 1 and cell 3). The numbers in the panels of A and B indicate the time in minutes. (**C**) FISH signals for rDNA were observed in the mutants after the two-step shift-up to 36°C. Cells fixed with paraformaldehyde were probed with rDNA for FISH and stained with DAPI. The frequencies of the observed phenotypes are indicated on the right-hand side. The *nda3* and *nda3 cut14* mutants were arrested in prometaphase at 20°C for 8 hours, released to 30°C for 9 minutes and further shifted to 36°C for 12 minutes. The *nda3 top2* mutants were temporarily released to 28°C for 12 minutes and then further shifted to 36°C for 15 minutes. Scale bars: 10 µm.

structure at anaphase (indicated by the arrows). This structure was presumably the non-segregated nucleolar structure. The thread-like structure (seen in cell 2 and cell 4) might contain partly segregated nucleolar chromatin (supplementary material Movie 7). These results indicated that most of the chromosomes were segregated, whereas nucleolar DNA was not segregated. In cell 2 of the *nda3 top2-191* strain, two initially separated nuclear portions were brought back to the center of the cell and merged together after telophase. The phenotypes in the two double mutants, *nda3*-cs *top2-342*-ts and *nda3*-cs *top2-191*-ts, were nearly identical. Therefore, Top2 seems to be continuously necessary until after the separation of the nucleolar region in late anaphase, regardless of the temperature-sensitive *top2* allele used. We also confirmed that the mutation in *top2-342* was G972D by sequencing; this mutation resides in the same DNA topoisomerase IV domain as does the mutation in *top2-191* (A801V) (Shiozaki and Yanagida, 1991). Consistent with this, these strains were originally isolated as mutants defective in the DNA-relaxing activity in vitro (Uemura and Yanagida, 1984). Thus, the phenotypes observed in this study were not allele specific.

Condensin and Top2 differentially affect chromosome segregation in anaphase

To compare the segregation defects caused by inactivation of condensin or Top2, the FISH method was applied using rDNA as a probe. Aberrant chromosomes produced in the two-step shift-up experiments, observed during late anaphase, were examined by FISH (Fig. 3C). In the control single-mutant *nda3* strain, the probe (a full-length 10.9 kb rDNA repeat unit) (Uzawa and Yanagida, 1992) showed that 100% of the cells had undertaken normal segregation by 10 minutes after the second shift-up to 36°C. For the double-mutant *nda3 cut14* cells, 68% of the cells had a visible lump structure at 12 minutes after the second shift-up. The lump did not hybridize with the rDNA probe, suggesting that rDNA had segregated in the condensin mutant. In 26% of the cells, however, rDNA did not segregate at all and was associated with the nuclear chromatin area. In sharp contrast, rDNA was not segregated in 71% of the *nda3 top2* double-mutant cells, and these cells exhibited balloon- or thread-like DNA signals (as in cell 1 or 2, Fig. 3B) at 10 minutes after the second shift-up. Intense rDNA FISH signals were located along the threads, strongly suggesting that the rDNA region was stretched in anaphase and later reversed to the middle of the cell in telophase. In 5% of the *nda3 top2* double mutants, rDNA remained in the middle of the cell, and the bulk of the chromosomes did not seem to segregate but to elongate. Taken together, the loss of condensin or Top2 in anaphase causes distinct defects in the segregation of rDNA in *S. pombe*, and in particular, the observed phenotypes heavily implicate Top2 in the segregation of rDNA in *S. pombe*.

Mutant condensin forms the lump containing telomere-adjacent DNA

In a previous study (Sutani et al., 1999), the wild-type condensin complex was found to localize briefly to the midzone of the cell during late anaphase. We suspected that the mutant lump structure was related to the presence of condensin at the cell midzone during late anaphase in wild-type cells. To localize the mutant condensin complex, GFP-tagged Cnd1 and Cnd2, the non-SMC subunits, and Cut3–GFP, the SMC4 subunit that binds to Cut14, were chromosomally integrated and expressed under their respective promoters in the control single mutant *nda3* (Cut14^{+}) and the double mutant *nda3 cut14* (Cut14^{-}). As shown in control *nda3* mutant cells cultured in the two-step shift-up experiment, midzone Cnd1–GFP signals were observed in late anaphase at 36°C (Fig. 4A). Notably, DAPI-positive signals that overlapped with these GFP signals were never observed in the control strain (n=22 cells examined), indicating that the midzone signals in the wild-type condensin background had no detectable DNA. Similarly, midzone Cut3–GFP (Fig. 4C) and Cnd2–GFP (Fig. 4E) signals without overlapping DAPI signals were also observed (n=32 and n=25, respectively). The strong GFP signals were also found in the segregated daughter nuclei until telophase, suggesting that the localization of condensin in late anaphase could be classified in two groups, nuclear and midzone.

In the *nda3 cut14* double mutant, we found that a DAPI-positive lump always colocalized with the GFP-tagged condensin subunits Cnd1, Cut3 and Cnd2 (Fig. 4B,D,F). The condensin subunit signals always emitted intense fluorescence in the lump. Examination of numerous cells revealed these signals always colocalized with DAPI (n=30, Cnd1–GFP cells inspected; n=27, Cut3–GFP cells; n=28, Cnd2–GFP cells) after exposure to 36°C for 12 minutes. The movement of the lump was dynamic, as seen in supplementary material Movie 4, and was not fixed at a particular site such as the midzone. We therefore concluded that the mutant condensin complex formed the lump that was bound to the DNA impaired in segregation. The mutant Cut14-208 protein also localized to the lump (data not shown), suggesting that the mutant condensin is defective at 36°C but still forms the condensin holocomplex.

Identification of the DNA regions that accumulated in the lump is of interest because the wild-type condensin at the midzone might have finished interacting with these regions before the completion of anaphase. It was clear that rDNA was not localized to the lump, as shown in Fig. 3C. In the *nda3 cut14* double mutant, we employed FISH using probes for centromeric DNA (Cen FISH) (Funabiki et al., 1993) and the centromeric protein CENP-A (Cnp1) tagged with GFP (Takahashi et al., 2000). We could not detect either Cen FISH containing pericentromeric probes or CENP-A, which recognizes the central centromere core, in the lumps, suggesting that the lump did not contain the centromere or kinetochore structure (Fig. 4G). By contrast, the GFP-tagged Taz1 protein (Chikashige and Hiraoka, 2001; Hayashi et al., 2009), which binds to telomeric repeats, was found to localize to >80% of the lumps (Fig. 4H, top panels). FISH signals produced by the telomere probe cos212 (Funabiki et al., 1993), which contains the telomere-adjacent DNA of chromosomes I and II, were also frequently observed at the lumps (Fig. 4H, bottom panels). Thus, condensin inactivation in late anaphase caused the missegregation of chromosome termini bound to mutant condensin. Whether DNA regions other than telomere-adjacent sequences are present in the lumps remains to be determined.

Condensin subunits are mitotically phosphorylated

To investigate how the post-transcriptional modification of condensin affects the mode of chromosome segregation, we next attempted to identify phosphorylation sites in mitotic condensin by mass spectrometry. Mitotic cells were obtained by employing the tubulin mutant *nda3-KM311*, which was chromosomally integrated with a FLAG-tagged wild-type *cut14*$^{+}$ gene (Cut14-3FLAG) under its native promoter (Nakazawa et al., 2008). Large-scale cultures of mitotic cells (5×10^{10} cells) were grown at 20°C for 8 hours, extracts were prepared and anti-FLAG antibodies were used to immunoprecipitate condensin holocomplexes. The precipitates

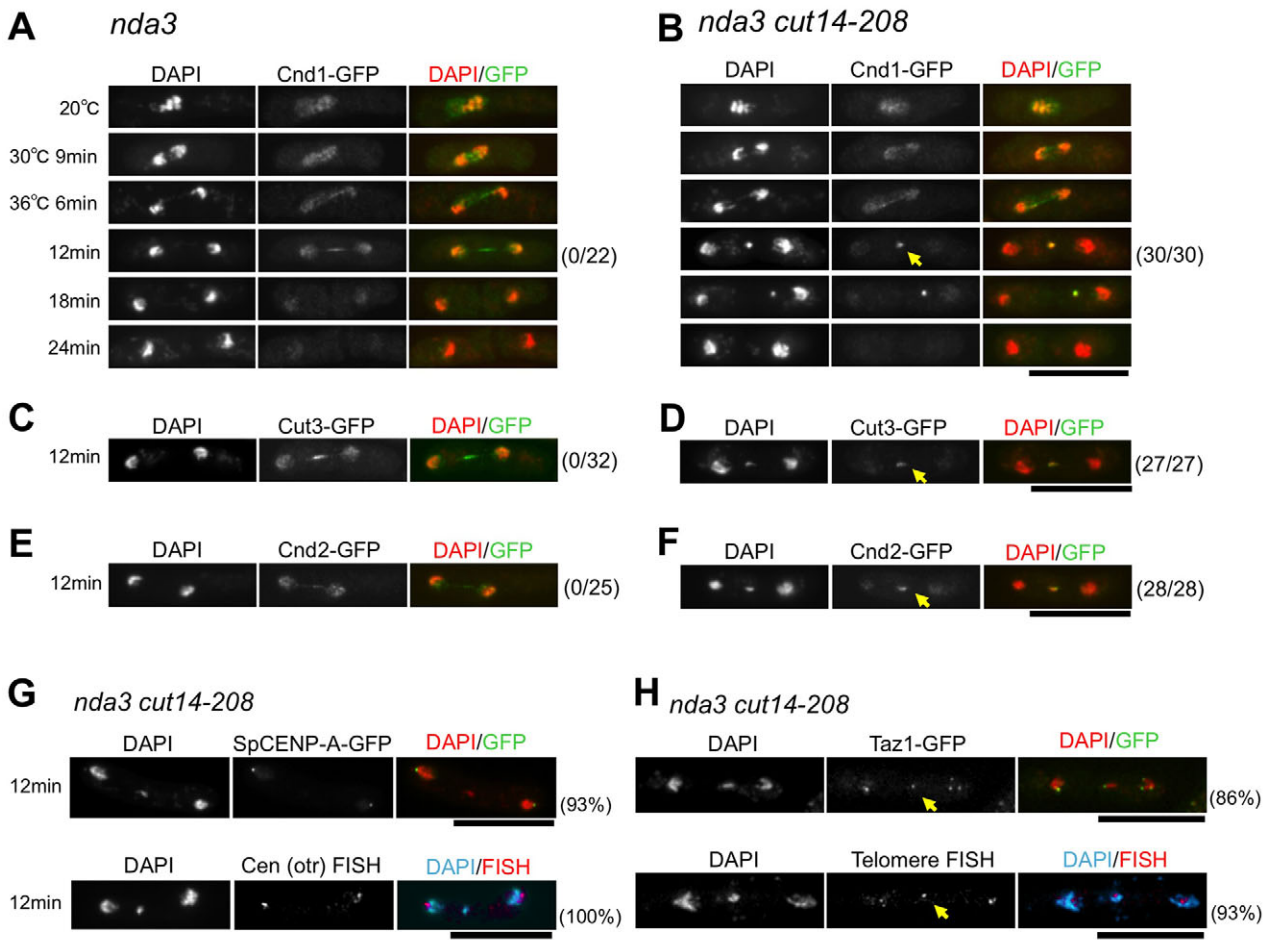

Fig. 4. The Cut14 mutant protein is present in a lump at late anaphase that also contains DNA. (**A**,**B**) GFP-tagged Cnd1, a non-SMC condensin subunit, was observed in the single *nda3* mutant (A) and the double *nda3*-cs *cut14*-ts mutant (B) at 20°C, 30°C and 36°C (the indicated times are after the shift-up). Cells were fixed with methanol. Cnd1–GFP was present in the lump, which also contained DNA in the double mutant (indicated by the arrow). The frequencies of cells with signals that colocalized with DNA are indicated in parentheses on the right-hand side. (**C**–**F**) The lump was observed in the *nda3 cut14* double mutant (D,F) when Cut3–GFP and Cnd2–GFP were used. (**G**) The *nda3 cut14* double-mutant cells expressing CENP-A–GFP (SpCENP-A-GFP) were observed after the two-step shift-up (top panel). The double-mutant cells, after the two-step temperature shift, were observed after pericentromeric (Cen) FISH analysis (bottom panel). (**H**) Double-mutant cells with an integrated Taz1–GFP gene were observed after the two-step shift-up (top panel). Double-mutant cells after the two-step shift-up were observed after FISH with the telomere-adjacent sequence probe (cos212) (bottom panel). The percentages of cells with signals that colocalized with DNA are indicated in parentheses on the right-hand side for G and H. Scale bars: 10 µm.

contained the five authentic subunits (Sakai et al., 2003; Sutani et al., 1999) (Fig. 5A), as compared with the silver-staining pattern of condensin isolated with the untagged control (Fig. 5B). The slices of in-gel tryptic digestion of mitotic condensin were subjected to mass spectrometry, and peptides derived from the heteropentameric subunits were identified (the coverage of peptides was 44–75%, supplementary material Fig. S2A).

We detected a total of 18 phosphorylation sites in mitotic condensin (five, two, zero, nine and two sites in the Cut3, Cut14, Cnd1, Cnd2 and Cnd3 subunits, respectively). Both SMC and non-SMC subunits were thus phosphorylated. Because the coverage of Cnd1 was only 44%, it remains to be determined whether Cnd1 was actually phosphorylated. The locations of the phosphorylated peptides in the amino acid sequences are shown in Fig. 5C for the Barren-like Cnd2 subunit and in supplementary material Fig. S2B–D for the other three subunits. Consistent with a previous report (Sutani et al., 1999), the Cut3 T19 residue, in the Cdc2 consensus site [T19]PDR, was phosphorylated.

Identification of putative Aurora-B-like Ark1 sites in the Cnd2 subunit

We focused on the putative phosphorylation sites of the Aurora-B-like kinase Ark1 of *S. pombe*, as the nuclear chromosomal recruitment of condensin requires the Ark1-associating protein Bir1 (also known as Cut17) (Morishita et al., 2001; Nakazawa et al., 2008). The kinase-dead mutation Ark1-K118R prevents the localization of condensin to chromosomes and has a strong dominant-negative effect on mitotic condensin (Petersen and Hagan, 2003). The kinase Aurora B is essential for the chromosomal localization of condensin in *Drosophila* (Giet and Glover, 2001) and *Caenorhabditis elegans* (Hagstrom et al., 2002). In vertebrates, Aurora B is required for enrichment of condensin II at kinetochores (Ono et al., 2004) and also for facilitating the localization of condensin I at mitotic chromosomes (Lipp et al., 2007; Takemoto et al., 2007). The consensus sequence of Aurora kinase (R/K-x-S/T-ϕ, where ϕ indicates a hydrophobic residue) (Cheeseman et al., 2002) was used to search for the putative Aurora target sites. The

five condensin subunits contained a total of 34 potential Aurora target sites, 23 of which were covered by the mass spectrometric analysis. Among these, three sites (S5, S41, S52) in the N-terminal region of the Barren-like Cnd2 subunit were indeed phosphorylated, as determined by mass spectrometry (Fig. 5C).

Preparation of a polyclonal antibody against a Cnd2 phosphorylated site

To detect phosphorylation of the Aurora kinase site, antibodies against the phosphopeptide [S45]ITPRRE-[S-*P*]-LNNS[S57], phosphorylated at S52, were raised (see Materials and Methods). The resulting antibodies were then used for immunoblotting of phosphorylated Cnd2 in *S. pombe* asynchronous (33°C, AS) and *nda3*-arrested (20°C, 9 hours, mitosis) extracts. As a negative control, we constructed *S. pombe* strains (*cnd2-A* or *cnd2-E*) in which the S52 codon was chromosomally replaced with alanine or glutamate codons (A and E) and tagged with GFP (see below). As shown in Fig. 6A, phosphorylated S52 was weakly detected in the asynchronously grown culture (at 33°C) but was more intense in mitotic extracts (20°C, 9 hours). However, phosphorylation was almost undetectable in extracts from the replacement mutants. Hence, we concluded that the phosphopeptide antibody was specific for S52-*P*, and that it intensely interacted with Cnd2 in *nda3* mitotic extracts. The other synthetic phosphopeptides tested did not produce appropriate antibodies.

Cnd2 S52 is phosphorylated throughout mitosis

To confirm that S52 phosphorylation peaked in mitosis, a block–release experiment was performed using the temperature-sensitive *cdc25-22* mutant (Fig. 6B). These cells were blocked in late G2 phase and released synchronously into mitosis by a temperature-shift from the restrictive 36°C to the permissive 26°C temperature. The timing of late anaphase or telophase was monitored by the increase in bi-nucleated cells without the septum. The G1–S phase corresponded to the increase in the number of bi-nucleated cells with a septum. The band for phosphorylated S52 was negligible in late G2 (0 minutes) but became intense and reached maximum intensity at 15–45 minutes after the release to 26°C. The peak of S52 phosphorylation coincided with the period from early mitosis to late anaphase and telophase. A weak, but still substantial, phosphorylated S52 band was detected at ~60 minutes, at the time corresponding to the onset of cytokinesis. We concluded that S52 phosphorylation was high and sustained throughout mitosis. By contrast, phosphorylation of Cut3 T19 by Cdc2 kinase, as detected by specific antibodies (Sutani et al., 1999), only briefly peaked at 15 minutes, corresponding to prophase and metaphase (Fig. 6B).

Inhibition of the analog-sensitive Ark1-as3 abolishes S52 phosphorylation

To determine whether phosphorylation of the Cnd2 S52 residue was dependent on Ark1, the antibody against S52-*P* was used to immunoblot mitotic extracts obtained from a strain with a chromosomal replacement of Ark1 with the *ark1-as3* allele (Hauf et al., 2007). This allele encodes an analog-sensitive protein that is specifically inactivated with 1-tert-butyl-3-naphthalen-1-ylmethyl-1H-pyrazolo[3,4-d]pyrimidin-4-ylemine (1NM-PP1) (Papa et al., 2003). The *ark1-as3* mutant was crossed into the *cdc25-22* Cnd2-8Myc strain. The resulting strain was then used for the Cdc25 block–release experiment using DMSO as the control (Fig. 6C, left-hand panels). To specifically inhibit Ark1 activity during mitosis, 5 μM 1NM-PP1 was added to the G2-arrested culture

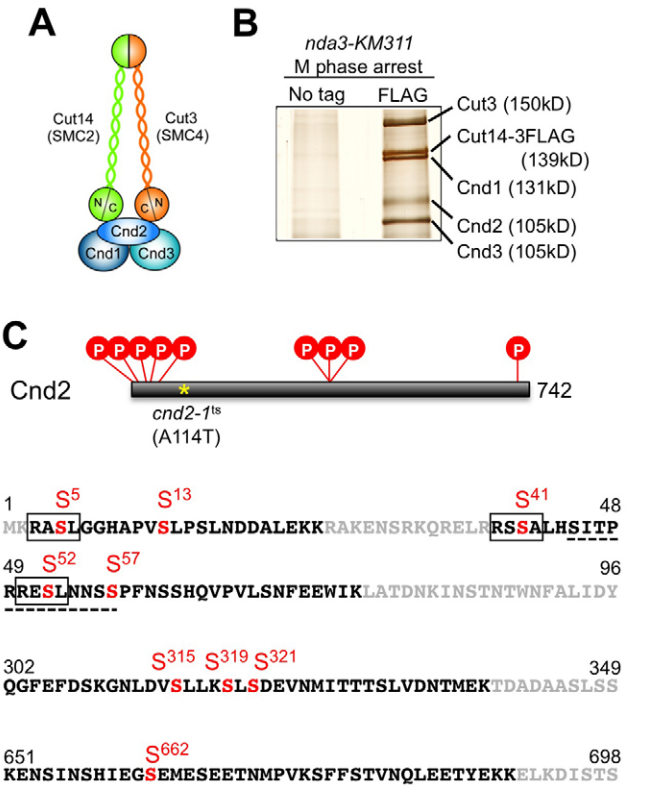

Fig. 5. Mitotic phosphorylation sites in condensin subunits. (**A**) A schematic showing condensin subunits in *S. pombe* and the name for SMC subunits. Cnd2 belongs to the Barren family. (**B**) The silver-stained SDS-PAGE pattern of mitotic condensin obtained by immunoprecipitation using antibodies against FLAG from extracts of arrested cold-sensitive *nda3-KM311* cells carrying Cut14–FLAG (FLAG). Extracts from the cold-sensitive *nda3-KM311* strain without the FLAG tag ('No tag') were used as a control. (**C**) The mass spectrometric identification of phosphorylation sites in Cnd2 (red S with residue numbers) is shown. The amino acid sequence underlined with the dashed line indicates the phosphopeptide containing S52-*P*, which was synthesized and for which a rabbit antibody was successfully obtained. Light gray sequences indicate peptides that were not recovered by mass spectrometry. The boxed sequences represent predicted sites for the kinase Aurora B (Cheeseman et al., 2002). The locations of the nine identified phosphorylation sites are shown in the cartoon, along with the *cnd2-1* mutation site.

(36°C) at 15 minutes before the release (26°C) into mitosis (Fig. 6C, right-hand panels). The S52-*P* residue of Cnd2 was detected during mitosis (from 30 to 90 minutes) in the presence of DMSO, whereas the S52-*P* band was not detected at all in the presence of 1NM-PP1. This result strongly suggests that Ark1 is responsible for the phosphorylation of the Cnd2 S52 residue. The addition of 1NM-PP1 increased the frequency of chromosome segregation defect displaying phi-shaped and streaked phenotypes, which also closely resembled the phenotype of the *cut17-275* mutant and of an Ark1 shut-off strain, confirming that Ark1 activity was inhibited by 1NM-PP1 (Hauf et al., 2007; Morishita et al., 2001; Petersen and Hagan, 2003). Mitotic progression was then quantitatively monitored by staining for the two types of the mitotic spindle (short spindle, <4 μm, and long spindle, >4 μm) with an anti-TAT1 antibody (Fig. 6C, bottom panels). The modes of spindle dynamics during the synchronous mitosis were basically identical in the presence or absence of 1NM-PP1.

Although Cnd2 S5 and S41 were phosphorylated, it was unknown whether phosphorylation was upregulated in mitosis and was diminished by 1NM-PP1. To clarify this issue, mass spectrometry was performed for Cnd2-3FLAG protein obtained from a *cdc25*-22 Cnd2-3FLAG *ark1-as3* culture that was released into anaphase in the presence or the absence of 1NM-PP1. For S5, seven peptides were obtained in the presence of DMSO, and two of them were phosphorylated (data not shown). In the presence of 1NM-PP1, nine peptides were obtained but none of them was phosphorylated. For S41, only one of eight peptides was phosphorylated in DMSO, but none of the nine peptides detected with 1NM-PP1 was phosphorylated. These results are not conclusive but suggest that there is an Ark1-dependent partial phosphorylation of S5 and S41. The peptide containing S52 was not covered in this experiment.

Ark1-dependent phosphorylation of Cnd2 is required for proper mitosis

To determine whether the three phosphorylated sites (S5, S41 and S52) are functionally relevant for Cnd2, we constructed multiple strains with replacements of these serine residues with alanine (A) and glutamate (E) as depicted in Fig. 7A. All integrated strains, except strains 3A and 2E-2, could grow and form colonies. The reason why integrants 3A and 2E-2 could not be obtained is unknown, but the 3A mutant is likely to be inviable (see below). Light microscopy of DAPI-stained A and E substitution mutants revealed substantial proportions of A mutant cells (but not of E mutants) with abnormal mitosis, particularly in late anaphase. The frequencies of abnormal anaphase were semi-quantitatively designated (Fig. 7A). Typical segregation abnormalities in different substitution mutant strains are shown in Fig. 7B.

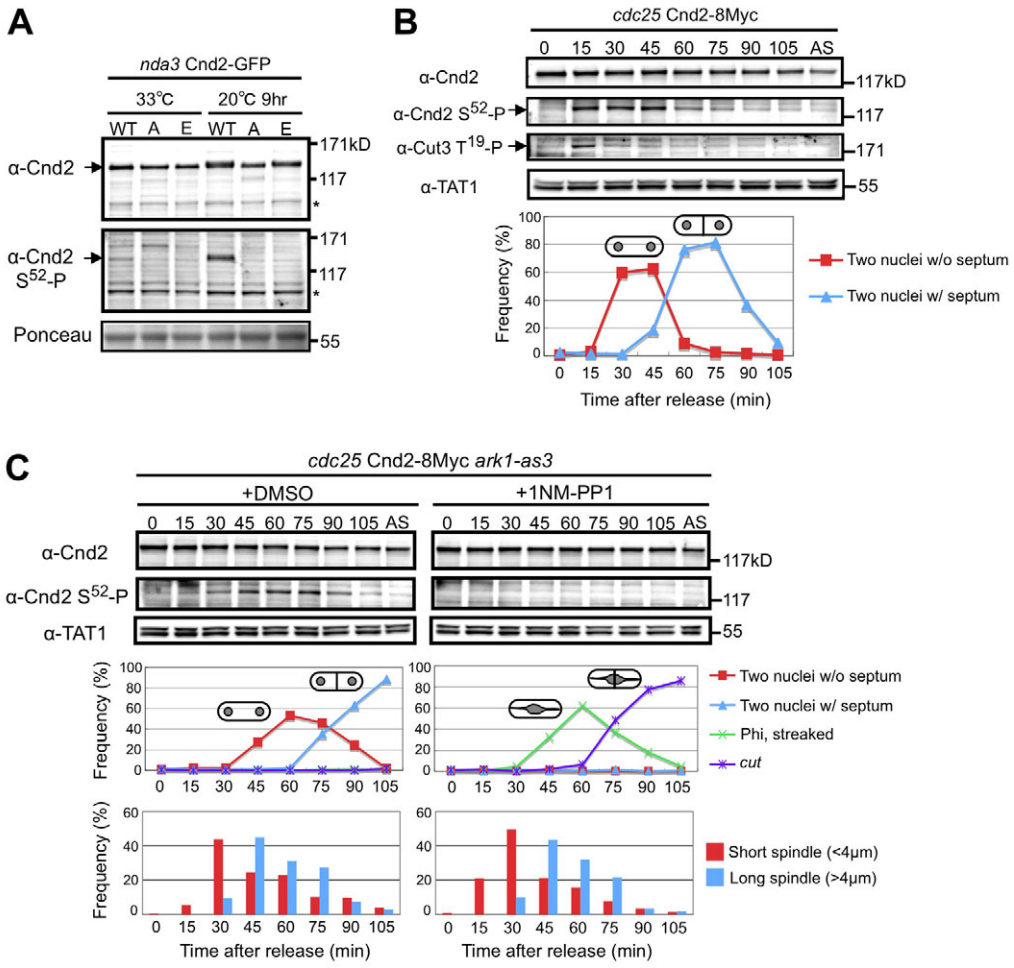

Fig. 6. Mitotically regulated phosphorylation of Cnd2 S52 by Ark1. (**A**) The specificity of polyclonal antibodies against the S52-*P*-containing phosphopeptide was examined. Asynchronous (33°C) and mitotic (20°C, 9 hours) extracts were prepared from cold-sensitive *nda3-KM311* Cnd2–GFP cells and were subjected to SDS-PAGE. Binding specificity was examined by comparison with wild-type (WT) Cnd2 with the substitution mutants A and E carrying the non-phosphorylatable alanine or phosphorylation mimetic glutamate residues. The arrow indicates the position of the Cnd2 band. Ponceau staining was used for the loading control of extracts. The asterisks indicate non-specific contamination bands. (**B**) Results of the block–release experiment using the *cdc25-22* Cnd2-8Myc strain. Cells were arrested at late G2 at 36°C and then released at 26°C. Both Cnd2 S52 and Cut3 T19 phosphorylation were detected (the arrows indicate band positions). The anti-TAT1 antibody against tubulin was used as a loading control. (**C**) A block–release experiment using the *cdc25-22* strain containing the two integrated markers, Cnd2-8Myc and the analog-sensitive *ark1-as3* allele (see the text for details). Two cultures with the control DMSO or the inhibitor (5 μM 1NM-PP1) were prepared. These compounds were added to the cultures 15 minutes before release into mitosis at 26°C. S52 phosphorylation was not detected in the culture treated with the inhibitor (top panel), which also showed aberrant segregation phenotypes displaying phi-shaped and streaked chromosomes (middle panel). Mitotic progression was quantitatively monitored by staining for the two types of the mitotic spindle (short spindle, <4 μm; and long spindle, >4 μm) with anti-TAT1 antibody (bottom panel). AS, asynchronous culture.

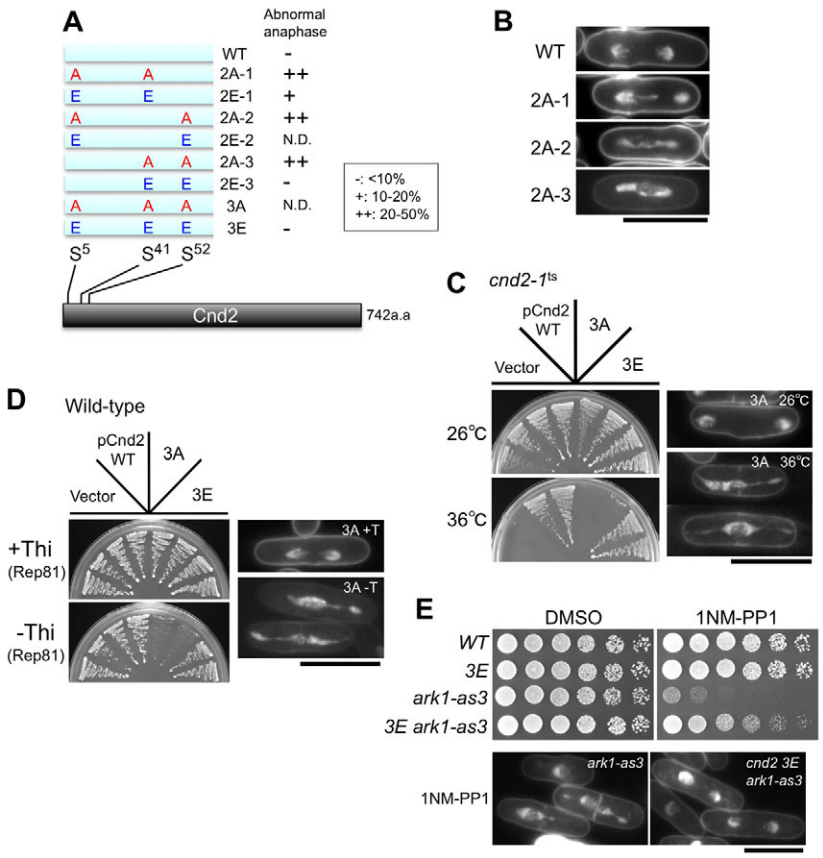

Fig. 7. *cnd2* mutants that cannot be phosphorylated by Ark1 are defective in segregation. (**A**) Multiple alanine and glutamate residue (A and E, respectively) substitutions were chromosomally integrated in the *cnd2* gene as indicated. The frequencies of abnormal anaphase were then analyzed. ++, 20–50%; +, 10–20%; –, <10%; ND, not determined (integrants were not obtained). (**B**) Micrographs of wild-type (WT), and substitution mutants 2A-1, 2A-2 and 2A-3 cells at telophase. (**C**) Plasmids carrying the wild-type *cnd2*$^{+}$ and substitution mutant genes were introduced into the temperature-sensitive *cnd2-1* mutant. The resulting transformants were plated at 26°C and 36°C. Micrographs of the 3A transformants are shown. (**D**) Mild overproduction of 3A had a dominant-negative effect on wild-type growth. The wild-type *cnd2*$^{+}$ (pCnd2 WT), and the mutant *cnd2* 3A and 3E genes, were placed under the inducible promoter *nmt* (plasmid REP81) and were repressed in wild-type cells in the presence of thiamine (+Thi; +T) or moderately overproduced in its absence (–Thi; –T). Vector, vector-only control. (**E**) Chromosomally integrating the *cnd2* 3E gene into the *ark1-as3* mutant allowed growth in the presence of 5 μM 1NM-PP1. The micrographs of *ark1-as3* single and 3E *ark1-as3* double-mutant cells in 1NM-PP1 are shown (bottom). Scale bars: 10 μm.

We then tested whether high-copy plasmids carrying the *cnd2* mutant genes under the native promoter could suppress the temperature-sensitive phenotype of the *cnd2-1* mutant (Aono et al., 2002). The plasmid carrying the 3A mutant gene failed to suppress the temperature-sensitive phenotype (Fig. 7C, left-hand panel), suggesting that the 3A mutant protein was not functional. The phenotypes of mutants carrying the 3A plasmid are shown in the right panel: severe condensation and segregation defects were observed at 36°C. Taken together with the failure to isolate the 3A substitution mutant, these results are consistent with the idea that the mitotic phosphorylation of the three Ark1 sites in the N-terminal region of the Barren-like Cnd2 subunit is absolutely essential for chromosome condensation and segregation.

The plasmid REP81 was used to overexpress the 3A or 3E substitution mutant proteins in the absence of thiamine (–Thi). In the presence of thiamine (+Thi), the promoter (REP81) used was shut off. As shown in Fig. 7D, the 3A mutant, but not the 3E mutant, caused a severe dominant-negative effect on colony formation. Micrographs of cells that overproduced the 3A mutant protein showed that they exhibited severe defects in chromosome condensation and segregation. The phenotypes strongly resembled those of the temperature-sensitive *cut14* and *cnd2* condensin mutants.

We examined whether the *cnd2* 3E substitution mutant produced the phenotype when Ark1 was inactivated. As shown in Fig. 7E, the *ark1-as3* single mutant was impaired in colony formation in 1NM-PP1, whereas the chromosomally integrated *cnd2* 3E *ark1-as3* double mutant considerably restored the growth in the presence of the drug. Consistent with this result, the anaphase segregation defect displaying phi-shaped chromosomes was observed in 34% and 95% cells for the double and single mutant, respectively. The single substitution *cnd2* S5E and S52E was combined with *ark1-as3*, and the drug sensitivity was also examined. S5E and S52E, but not S5A and S52A, clearly alleviated the sensitivity of *ark1-as3* in the presence of 3 μM 1NM-PP1 (supplementary material Fig. S3A,B). Cnd2 S5E and S52E mutants, but not S5A and S52A, thus become independent of Ark1 inactivation. We then examined the temperature-sensitive phenotype of the double mutants with *cnd2* S5 or S52, and *cut17-275*, which is in the Aurora-associated subunit survivin (supplementary material Fig. S3C,D). The *cut17* and S5A or S52A double mutants were synthetic lethal, but the combination with S5E or S52E could form colonies at the semi-permissive temperature.

Discussion

Here, we examined how condensin and Top2 function after the completion of condensation, or even after anaphase, using *S. pombe* cold-sensitive tubulin and temperature-sensitive condensin or *top2* mutant cells. At 20°C, the inactivated mutant tubulin causes a reversible arrest at an early mitotic stage owing to the absence of the spindle, whereas temperature-sensitive condensin and temperature-sensitive Top2 are functional. At 36°C, the mutant tubulin is activated, leading to the rapid formation of the spindle, allowing the cell to segregate the mitotic chromosomes in a highly synchronous fashion, while the temperature-sensitive proteins become inactivated. Using the intermediate temperature (28–30°C for 7–10 minutes) that is permissive for both cold-sensitive and temperature-sensitive proteins, we were able to inactivate condensin and Top2 after anaphase by a further temperature shift to 36°C. The experimental system described here is unique to *S. pombe*, as such a cold-sensitive tubulin mutation has not been made in other organisms.

We performed two sets of temperature shift-up experiments to inactivate condensin or Top2 after condensation or anaphase, and we examined the mutant phenotypes in detail. If the role of condensin or Top2 in mitotic chromosomes was already completed before inactivation, subsequent steps should occur normally. The one-step shift-up experiments for inactivating condensin or Top2 after condensation showed that both condensin and Top2 are required for the subsequent segregation, consistent with the previous work of Wignall et al. and Uemura et al. (Wignall et al., 2003; Uemura et al., 1987). We then performed a two-step shift-up to inactivate condensin or Top2 after late anaphase, and our results established that Cut14 and Top2 are required even during telophase, where they seem to be needed for keeping telophase chromosomes apart.

Severe segregation defects were caused by inactivation of Top2 after condensation, in that sister chromatid separation did not seem to occur at all. By contrast, in the *cut14* condensin mutant, the bulk of chromosomes (and the nucleolus) were already separated or extended in anaphase, although their structures were often reversed to the unsegregated state in telophase. The segregation of the rDNA-containing nucleolus was strongly blocked in *top2* but not in *cut14* mutant cells, when inactivation occurred after condensation or anaphase. A possible interpretation of these results is that sister chromatids were largely pre-separated during condensation, but they were brought back at least partly to the non-separated state when condensin was inactivated. In other words, condensin was continuously required until telophase to maintain the separated chromosome structures. In *top2* mutant cells, topological constraints (unfavorable supercoiling or catenation) that are probably continuously produced during mitosis were not resolved, resulting in a failure in chromatid separation. However, anaphase chromosomes did not apparently return to the decondensed state in the *top2* mutant, suggesting that condensin plays a large role in maintaining the condensed state from anaphase to telophase. Thus, condensin and Top2 are needed in distinct ways, although they both function throughout mitosis. Condensin continues to maintain the status of compacted and pre-separated chromosomes, whereas Top2 removes topological constraints continuously generated during mitosis. The rDNA regions might produce abundant topological constraints after condensation in *S. pombe*.

We do not know whether individual condensin complexes continuously associate with the same chromosome site throughout mitosis (in other words, whether condensin molecules act in a processive way during mitosis) or, alternatively, whether the mitotic role of condensin is carried out by a distributive association process; this is an important but unanswered issue. We speculate that condensin, at least in part, might act in a distributive fashion during mitosis, because condensin localization patterns on chromosomes exhibit rapid changes from mid-mitosis to late mitosis (Nakazawa et al., 2008). Top2 might also act in a distributive manner in *S. pombe* because of its ability to catalyze the mending of numerous lesions that produce topological constraints.

Mitotic condensin is highly phosphorylated: 18 sites were found in the ~50% coverage sequence of the five subunits assayed by mass spectrometry. We focused on the three phosphorylated sites in the N-terminal region of Cnd2 (the Barren-like subunit), all of which belonged to the Aurora-B kinase consensus sequence. *S. pombe* Ark1 is the catalytic subunit of an Aurora-B-like kinase complex [which also comprises the survivin-like protein Bir1 (Cut17), INCENP-like Pic1 and Borealin-like Nbl1] (Bohnert et al., 2009; Leverson et al., 2002; Petersen et al., 2001) that behaves like a chromosome passenger complex (CPC) (Ruchaud et al., 2007). We succeeded in detecting the Ark1-mediated phosphorylation of S52 in Cnd2 by using an analog-sensitive *ark1* mutant strain. Ark1 is known to be involved in regulating mitotic events from spindle assembly to cytokinesis (Leverson et al., 2002; Petersen et al., 2001), consistent with the timing of Cnd2 S52 phosphorylation.

The differential timing of phosphorylation of Cnd2 S52 and Cut3 T19 is remarkable, clearly indicating that there are differential contributions of Cdk1 (Cdc2) and Aurora B (Ark1) to condensin regulation. Cdk1 ensures the nuclear mobilization of condensin upon the entry into mitosis (Sutani et al., 1999). Condensin remains localized to chromosomes after Cdk1 inactivation, perhaps because Aurora-B-mediated phosphorylation of Cnd2 S52 is sustained until telophase. Aurora B ensures docking of chromatin to condensin and maintains condensin in association with chromatin, whereas Cdk1 enables condensin to enter the mitotic nucleus (Nakazawa et al., 2008). In this study, a non-SMC subunit was shown to be the target of regulation by Aurora B. This finding implies that the non-SMC trimer that binds to the ATPase domains of SMC subunits regulates the actual mitotic function of condensin. Alternatively, the non-SMC subcomplex directly interacts with condensed mitotic chromatin.

Our analysis using multiple alanine or glutamate residue substitutions in the three phosphorylation sites at the N-terminal region of Cnd2 strongly suggested that Ark1-mediated phosphorylation of Cnd2 is required to regulate condensin throughout mitosis. The alanine mutants, but not the glutamate mutants, showed mitotic defective phenotypes similar to those of the temperature-sensitive condensin mutants. The mitotic function of condensin might thus be mediated by phosphorylation by the Ark1 Aurora kinase on the non-SMC Barren-like Cnd2 subunit. Because the consensus target sequence for Aurora is not strict, it remains to be determined whether these sites are redundantly phosphorylated by other kinases. Indeed, as an example, protein kinase A (PKA) prefers R-R/K-x-S/T, R-x-x-S/T or R-x-S/T as target sequences (Kennelly and Krebs, 1991), and this sequence overlaps with that of Aurora kinases (Cheeseman et al., 2002). Condensin might be phosphorylated at the same sites by different kinases in different cell cycle phases, such as mitosis and interphase, or under different environmental and nutritional conditions. Thus, Aurora might be one of multiple kinases that affect condensin.

The lumps formed in condensin mutants shed light on how segregation failed in these mutants, particularly during late anaphase and telophase. The lumps contained concentrated mutant condensin bound to DNA and definitively contained telomeric DNA and protein. The presence of mutant condensin and telomeric components in the lumps suggests that the role of condensin is to keep chromosomal ends apart before cytokinesis. Chromosomal ends might need abundant condensin in order to keep them apart in late anaphase and telophase, the final step of chromosome segregation. A recent interesting report (Motwani et al., 2010) describes the connection of telomere protein Ccq1 (Flory et al., 2004; Miyoshi et al., 2008; Tomita and Cooper, 2008) with condensin proteins Cut3 and Cut14 proteins; defects in Ccq1 causes an entangled telomere, which is rescued by overproduction of Cut3 and Cut14 proteins. Condensin might have a specific role for telomere separation in anaphase. Alternatively, condensin in late anaphase might become a signaling molecule to initiate cytokinesis, and cytokinesis might be coupled with the finishing of segregation. Note that condensin is present in the midzone during late anaphase in the wild-type cells.

Materials and Methods

Strains, plasmids and media

The *S. pombe* haploid wild-type strain 972 h^- and its derivative mutant strains, including the temperature-sensitive *cut14-208* (Saka et al., 1994), temperature-sensitive *top2-191*, temperature-sensitive *top2-342* (Uemura and Yanagida, 1984), temperature-sensitive *cnd2-1* (Aono et al., 2002), temperature-sensitive *cut17-275* (Morishita et al., 2001) and cold-sensitive *nda3-KM311* (Hiraoka et al., 1984), were used. Site-directed PCR-based mutagenesis was used to construct several *cnd2* alanine or glutamate mutants. Briefly, complementary pairs of oligonucleotide DNAs with mutations were used as PCR primers, followed by two rounds of PCR. The mutated *cnd2* gene was cloned and chromosomally integrated together with the tagged GFP, the $adh1^+$ 3′ non-coding region and the kanamycin-resistant gene kan^{R+} into the endogenous *cnd2* locus of the h^- *leu1* (HM123) strain. Correct integration was confirmed by PCR and digestion of the PCR products with restriction enzymes. The chromosomally integrated strains with epitope-tagged (GFP, Myc_8 and FLAG) Cut14, Cut3, Cnd2, Cnd1 and Cnp1 (*S. pombe* CENP-A) were as previously described (Aono et al., 2002; Nakazawa et al., 2008; Sutani et al., 1999; Takahashi et al., 2000). The strain with GFP-tagged Taz1 was provided by the Yeast Genetic Resource Center (YGRC). For the RFP-tagged histone H2A strain, a fragment containing the $hta1^+$ gene with RFP and the $ura4^+$ gene at the C-terminus was constructed and integrated into the $leu1^+$ locus of an h^+ *ura4* strain. Correct integration was confirmed by PCR using genomic DNA from Leu^- Ura^+ transformants. The *ark1-as3* strain was a gift from Silke Hauf (Friedrich Miescher Laboratory of the Max Planck Society, Tuebingen, Germany). 1NM-PP1 (Calbiochem) was diluted with DMSO, and a 1 mM stock solution was used to inhibit the Ark1-as3 protein (Hauf et al., 2007). The pRep81-cnd2 wild-type, 3A and 3E plasmids were derived from the plasmids used for chromosomal integration. The culture media used for *S. pombe* were complete YPD, SPA sporulation medium and minimal EMM2 medium (Saka et al., 1994). Cells were counted using a Sysmex F-800 (TOA Medical Electronic).

Synchronous culture and temperature shift experiments

Temperature shift experiments with the *nda3-KM311 cut14-208* or the *nda3-KM311 top2-191* double mutants were performed as follows. The cold-sensitive, or cold-sensitive and temperature-sensitive, cells were grown in YPD at 30°C (for the *nda3 cut14*) or 28°C (for the *nda3 top2*). When cell concentrations reached 4×10^6 cells per ml, the cultures were transferred to 20°C and incubated for 8 hours to reversibly block spindle formation and to arrest cells at an early mitotic stage (Hiraoka et al., 1984). Cells were then released to 36°C to allow spindle formation and cell cycle progression. After this transfer, cell aliquots were taken at several time-points and fixed with 2.5% glutaraldehyde. For two-step temperature shifts, cells were released to 30°C (for the *nda3 cut14*) or 28°C (for the *nda3 top2*) for spindle formation without inactivation of the temperature-sensitive protein. After incubation at these semi-permissive temperatures for 9 minutes (for the *nda3-cut14*) or 12 minutes (for the *nda3-top2*), cells were released to 36°C for inactivation of the temperature-sensitive protein. For the block and release experiment with the *cdc25-22* mutant (Moreno et al., 1989), cells were grown in YPD at 26°C (to 3×10^6 cells per ml) and then shifted to 36°C for 4.25 hours to block cells at the late G2 phase. Cells were then released to 26°C, and aliquots were taken every 15 minutes for immunoblotting and measurement of the septation index.

Live cell analysis

Live imaging of the *nda3*-cs *cut14*-ts or the *nda3*-cs *top2*-ts double mutant expressing histone-H2A–RFP was performed as follows. Small aliquots of mitotically arrested cells at 20°C were released to a test tube and incubated at 36°C for 1 minute. When the temperature shift was performed in two steps, cells were incubated at 30°C for 7 minutes (for *nda3 cut14*) or at 28°C for 10 minutes (for *nda3 top2*). These incubation times at semi-permissive temperature were shortened by 2 minutes compared with those in the fixation experiments to allow time for specimen preparation. Cells were then concentrated to 5×10^6–10×10^6 cells per ml by brief centrifugation and mounted onto a glass slide with a coverslip or poured into a microfluidic plate (CellASIC). Immediately after the mounting or pouring of cells, time-lapse recording was started. Time-lapse images were recorded by three-dimensional microscopy using a DeltaVision system (Applied Precision, Seattle, WA). Oil-immersion objective lenses were used (PlanApo 60×, NA 1.4; Olympus). Three optical sections were collected at 0.5 minute intervals at 36°C. The vertical separations between these sections were 0.5 µm. Image projection and deconvolution were performed using an imaging workstation (SoftWoRx; Applied Precision).

Fluorescence microscopy and FISH

Procedures for DAPI staining, fluorescence microscopy and FISH were as previously described (Funabiki et al., 1993; Hagan and Hyams, 1988; Uzawa and Yanagida, 1992). For methanol fixation, cells were harvested by filtration and soaked in 100% methanol for 30 minutes at –80°C. Then, the solution containing the cells was gradually diluted with 30% methanol in PBS, and the cells were washed three times with PBS. The all-in-one microscope BZ9000 (Keyence, Japan) was used to observe the fixed cells.

Immunochemistry

Protein extracts were prepared by cell breakage using glass beads in extraction buffer (25 mM Tris-HCl pH 7.5, 0.1% NP-40, 10% glycerol and 1 mM DTT) supplied with protease inhibitor cocktail (Sigma). The extracts were boiled with LDS sample buffer and loaded onto a custom-made 3–8% gradient Tris-Acetate gel (NuPAGE, Invitrogen). Immunoblotting was performed using antibodies against the following proteins: Cnd2 (Aono et al., 2002), Cut3 T19-*P* (Sutani et al., 1999), TAT1 (a gift from Keith Gull, University of Oxford, UK). The Cnd2 S52-*P* antibody was obtained by immunizing rabbits with the synthetic phosphopeptide ([S45]ITPRRE-[S-*P*]-LNNS[S57]; Sigma).

Identification of phosphorylation sites by mass spectrometry

To arrest cells in mitosis, the *nda3-KM311* Cut14-3FLAG strain was cultured at 20°C for 8 hours. Aliquots of 5×10^{10} cells were collected and frozen in liquid nitrogen with extraction buffer (25 mM Tris-HCl pH 7.5, 15 mM EGTA, 15 mM $MgCl_2$, 0.1% NP-40, 60 mM β-glycerophosphate, 15 mM *p*-nitrophenylphosphate, 0.5 mM Na_3VO_4, 0.1 mM NaF, 1 mM PMSF, 1 mg/ml aprotinin and 1 mM DTT). Frozen cells were disrupted with a Retsch mechanical grinder in the presence of liquid nitrogen. Cell pellets were centrifuged at 7000 rpm (10,000 ***g***) at 4°C and these supernatants were further centrifuged at 20,000 rpm (36,000 ***g***). FLAG M2 agarose beads (Sigma) were added to the clarified supernatant and rotated at 4°C for 2 hours. The beads were washed four times with extraction buffer, and immunoprecipitated proteins were eluted with the FLAG peptide (Sigma). The eluted proteins were concentrated with 10% TCA and washed twice with ice-cold acetone. After the addition of LDS sample buffer and boiling, samples were separated by SDS-PAGE (12.5% gel). After in-gel digestion with modified trypsin (Roche), the resulting peptides were analyzed by online liquid chromatography-tandem mass spectrometry on a Finnigan LCQ Advantage (Thermo Fisher). All tandem mass spectra were searched against the *S. pombe* non-redundant protein database, including common contaminants such as trypsin and keratin, with the Mascot program (Matrix Science, London, UK).

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