CENP-C Functions as a Scaffold for Effectors with Essential Kinetochore Functions in Mitosis and Meiosis

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SUMMARY

The conserved kinetochore protein CENP-C plays a fundamental role in chromosome segregation, but its specific functions remain elusive. We have gained insights into the role of CENP-C through identification of interacting effector proteins required for kinetochore function in fission yeast. Fta1/CENP-L is a primary effector that associates directly with Cnp3/CENP-C, and ectopic localization of Fta1 largely suppresses the mitotic kinetochore defects of cnp3Δ cells. Pcs1 functions downstream of Cnp3 to prevent merotelic attachment. In meiosis, Cnp3 further associates with and recruits Moa1, a meiosis-specific protein exclusively required for the mono-orientation of kinetochores. Genetic and biochemical analyses identified Cnp3 mutants that preserve intact mitotic kinetochore function but abolish the association with Moa1 and meiotic mono-orientation. Overall, therefore, our studies identify effectors of CENP-C in mitosis and meiosis and establish the concept that CENP-C serves as a scaffold for the specific recruitment of essential kinetochore proteins.

INTRODUCTION

Faithful segregation of the replicated genome during cell division in eukaryotes depends on the proper attachment of sister kinetochores to the spindle microtubules in correct orientation. During prometaphase, spindle microtubules search for and associate with kinetochores, large proteinaceous structures organized on centromeres that act as an interface for microtubule-chromosome attachment. In mitosis, kinetochores on sister chromatids are finally captured from opposite poles (bipolar attachment). In contrast, during the first meiotic division (meiosis I), sister kinetochores within one homolog attach to microtubules that emanate from the same spindle pole (monopolar attachment) and homologous chromosomes, but not sisters, are segregated at anaphase I. Kinetochores are thus regulated differently between mitosis and meiosis (Hauf and Watanabe, 2004).

So far, more than 80 kinetochore proteins have been identified. Among them, CENP-A and CENP-C are the components that possess all the properties of DNA binding at centromeres, which is essential for kinetochore function and conservation in all eukaryotes (Cheeseman and Desai, 2008; Maney et al., 2000; Talbert et al., 2004). CENP-A is a well-characterized histone H3 variant present solely in the centromeric nucleosome; its primary role lies in marking the centromere because no aspects of kinetochore assembly and centromeric function are seen when CENP-A is absent (Cleveland et al., 2003; Howman et al., 2000). CENP-C is also closely associated with active centromeres (Fukagawa and Brown, 1997; Fukagawa et al., 1999; Kalitsis et al., 1998; Tomkiel et al., 1994) and binds directly to centromeric DNA (Sugimoto et al., 1994; Yang et al., 1996), although it is not absolutely required for kinetochore assembly like CENP-A (Ando et al., 2002; Foltz et al., 2006; Saitoh et al., 1992; Westermann et al., 2003). In C. elegans, loss of HCP-4 (a CENP-C homolog) expression results in a failure of sister centromeres to resolve and form a back-to-back sister kinetochore configuration in mitosis, suggesting that CENP-C proteins have a role in preventing merotelic attachment and promoting the proper geometry of kinetochores (Moore and Roth, 2001). Recently, a new complex, CENP-T/W, was identified in vertebrates as another DNA-binding kinetochore component that may act in parallel with CENP-C (Hori et al., 2008). Despite the paramount importance of CENP-C in kinetochore function, the characteristic or specific molecular role of CENP-C remains largely elusive because of the pleiotropic abnormalities of kinetochores in CENP-C-depleted cells.

In this study, we report a comprehensive dissection of the role of Cnp3, a fission yeast CENP-C homolog. We identify three Cnp3 associating factors, Fta1, Pcs1 and Moa1, whose centromeric localization is Cnp3 dependent. We analyze the relevance of these interactions to faithful chromosome segregations in mitosis and meiosis and define them as CENP-C effectors in fission yeast.

RESULTS

Characterization of Cnp3/CENP-C-Deleted Fission Yeast

To explore the molecular function of CENP-C, we analyzed Cnp3 (a CENP-C homolog) in fission yeast, a genetically tractable model organism that has centromeres with a simple but
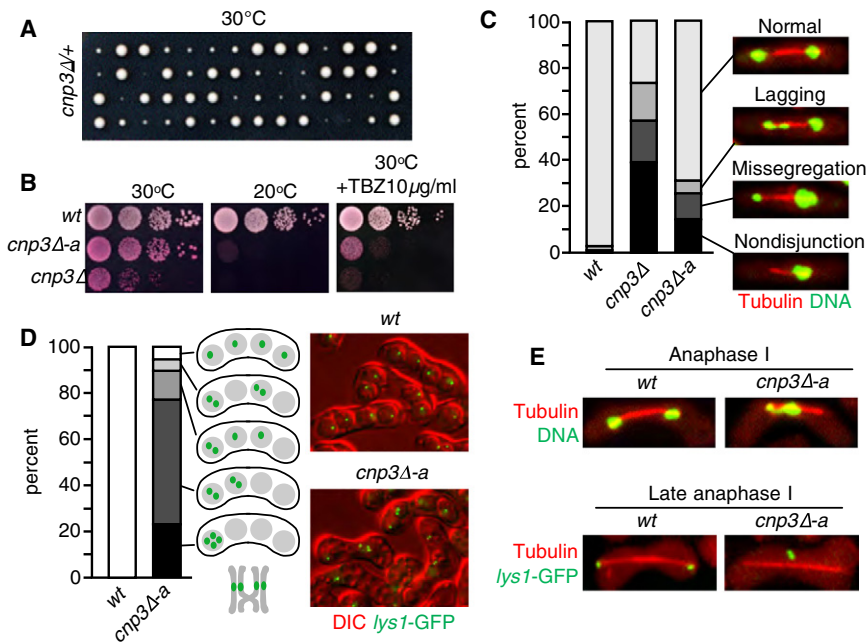
Figure 1. Characterization of cnp3Δ

(A) Spores from cnp3Δ/+ diploid strain were tetrad dissected on a YES plate and incubated at 30°C.

(B) cnp3Δ cells grow poorly and are sensitive to cold and TBZ. Wild-type, better-growing cnp3Δ-a, and poor-growing cnp3Δ cells were spotted onto YES plates containing Phloxine B, then incubated under the indicated conditions.

(C) Chromosome segregation was observed in anaphase in the indicated cells (n > 100). In cells grown at 30°C, a spindle was visualized by the mCherry-tagged α-tubulin (atb2[+]) (red), and DNA was stained with Hoechist33342 (green).

(D) Homothallic cells marked with lys1-GFP were sporulated at 30°C, and meiotic segregation of lys1-GFP was scored in four-spored asci (n > 200).

(E) Homothallic cells were induced to meiosis at 30°C. The spindle was visualized by the mCherry-tagged α-tubulin (red) and DNA was stained with Hoechist33342 (green). Representative pictures at anaphase I are shown. Note that centromere-associated lys1-GFP signals are not along the spindle in the cnp3Δ-a cell, indicating that they failed to attach microtubules.

well-conserved constitution (Pidoux and Allshire, 2004). We replaced the cnp3[+] gene in a diploid strain with the kanMX6 cassette. The obtained heterozygous disruptants (cnp3Δ/+) were sporulated and subjected to tetrad analysis. In contrast to the previous report (Holland et al., 2005), a majority of cnp3 null spores could germinate and propagate (Figure 1A). We do not know the reason for this difference, but it might originate from the different growth conditions. The cnp3Δ colonies grew slowly and stained dark on a plate containing the vital stain Phloxine B, indicating that cnp3Δ cells frequently die during division (Figure 1B). In addition, cnp3Δ cells were extremely sensitive to cold and the microtubule destabilizing drug thiabendazole (TBZ) (Figure 1B), characteristics linked to defects in kinetochore microtubule attachment. We noticed that better-growing colonies frequently emerged when poor-growing cnp3Δ colonies derived from tetrad dissection were restreaked and that the property of better growth was inherited stably during proliferation (see Figure S1A available online). Backcrossing of these better-growing cnp3Δ-a ("a" denotes adapted) cells and subsequent tetrad dissection revealed them to carry an extragenic suppressor (Figure S1B). To identify the suppressor mutation in cnp3Δ-a cells, we performed genome-wide sequencing and found that cnp3Δ-a carries a mutation in the pst2[+] gene (pst2-G745R), which cosegregates with the suppression (Figures S1C and S1D). These data indicate that fission yeast CENP-C-depleted cells are barely viable but not lethal, albeit they readily acquire extragenic suppressor(s) during proliferation (see Discussion).

Since CENP-C is an essential component of the kinetochore in budding yeast, flies, and vertebrates, the phenotypes observed in fission yeast cnp3Δ (poor growth and cold sensitivities) might be caused by kinetochore defects. Indeed, primary cnp3Δ cells, which are cultured immediately after tetrad dissection and, therefore, are mostly free from suppressor mutations, display lagging chromosomes (∼15%) as well as a high incidence of

unequal nuclear division (missegregation, ∼20%) and even complete nondisjunction (nondisjunction, ∼40%) in anaphase, indicative of an extensive defect in kinetochore-microtubule attachment (Figure 1C). The better-growing cnp3Δ-a cells that carry the pst2-G745R mutation still preserve severe cold and TBZ sensitivities (Figure 1B) and abnormal chromosome segregation (Figure 1C and Figure S2A), meaning that the defects observed in the cnp3Δ-a cells represent those of the original cnp3Δ cells at the minimum. Importantly, the pst2-G745R mutation itself shows few defects in mitotic or meiotic kinetochore function (Figures S1E and S1F), rationalizing that the observed defects of cnp3Δ-a are largely attributable to cnp3Δ. Therefore, when stable cnp3Δ cells were required for further analysis such as during meiosis, we used cnp3Δ-a cells to prevent the accumulation of various spontaneous suppressor mutations within the population.

The requirement of Cnp3 for meiotic chromosome segregation was examined by inducing meiosis in homothallic cnp3Δ-a cells and counting the spore number. In wild-type cells, almost all zygotes produced four spores, whereas nearly half of cnp3Δ-a asci contained irregular numbers of spores, suggesting that aberrant meiotic chromosome segregation took place (Figure S2B). Defects in meiotic chromosome segregation were further delineated by observing the segregation pattern of lys1-GFP signals in four-spored asci (Figure 1D). Strikingly, more than 75% of cnp3Δ-a four-asci displayed nondisjunction of homologs (all signals on either side of asci) at meiosis I. Accordingly, inspection of spindle and DNA or centromeres at anaphase I revealed the defective attachment of kinetochores to microtubules (Figure 1E). Significant numbers (∼12.5%) of asci showed a 2:1:1:0 pattern of segregation (Figure 1D), indicating that the mono-orientation of sister chromatids at meiosis I was defective as well. Taken together, these results suggest that fission yeast Cnp3 plays crucial roles in the attachment and orientation of kinetochores in meiosis. Since a suppressor mutation itself
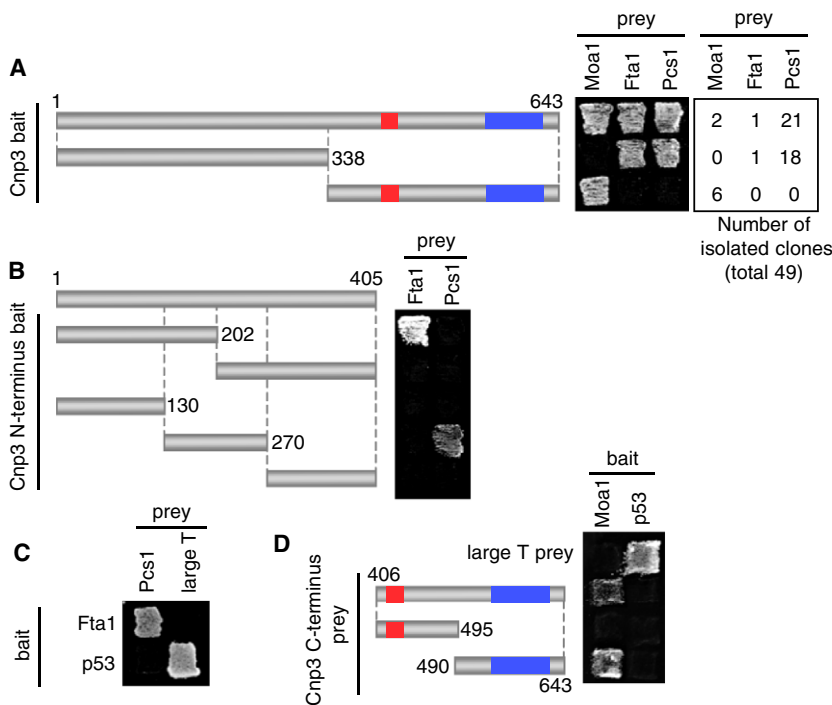
Figure 2. Cnp3 Interacts with Fta1, Pcs1, and Moa1

(A) Fta1, Pcs1, and Moa1 were identified as Cnp3-interacting proteins by yeast two-hybrid screening. (Left) Full-length, N-terminal, or C-terminal fragments of Cnp3 used as bait for two-hybrid screening are shown. The CENPC motif and Mif2-homologous region are shown as red and blue rectangles, respectively (Talbert et al., 2004). (Middle) The indicated sets of bait and prey were cotransformed into the tester strain AH109 and their interaction was detected on selective (SD-Trp-Leu) plate. (Right) The number of clones isolated by screening is summarized.

(B) The interaction domains for Fta1 and Pcs1 in the N-terminal region of Cnp3.

(C) Fta1 and Pcs1 interact in yeast two-hybrid assay. The large T antigen and p53 pair was used as a positive control.

(D) The interaction domain for Moa1 in the C-terminal region of Cnp3.

does not impair meiotic chromosome segregation (see Discussion), we reason that these defects are largely attributable to *cnp3Δ* itself.

Identification of Interacting Molecules with Cnp3

To obtain further insight into the molecular function of CENP-C, we searched for Cnp3-interacting proteins by yeast two-hybrid screening. We carried out screening against the two-hybrid prey library made from fission yeast cDNA using full-length, N-terminal, or C-terminal fragments of Cnp3 as bait (Figure 2A). A total of 49 positive clones were obtained from $\sim7.5 \times 10^6$ transformants. Among them, 41 clones associated with the N-terminal fragment of Cnp3 but not with the C-terminal fragment, and 8 clones associated with the C-terminal but not the N-terminal fragment. Sequence analysis of the obtained plasmids revealed that the former 41 clones encoded either *fta1⁺* (Liu et al., 2005) or *pcs1⁺* (Rabitsch et al., 2003), while the latter 8 clones all encoded *moa1⁺* (Yokobayashi and Watanabe, 2005) (Figure 2A). Further truncation of the Cnp3 N-terminal domain revealed that the interaction domains of Fta1 and Pcs1 partly overlap but are separable (Figure 2B). Intriguingly, Fta1 and Pcs1 themselves interact in the yeast two-hybrid assay (Figure 2C), suggesting that Fta1, Pcs1, and the N-terminal domain of Cnp3 associate closely with one another. Analysis using truncated versions of the C-terminal Cnp3 suggested that the C-terminal region of Cnp3 (490–643), which contains the Mif2-homologous domain but not the CENPC motif, is important for the association with Moa1 (Figure 2D).

Deletion analysis of the Cnp3 protein together with the replacement of conserved residues revealed that some conserved amino acid sequences in both the CENPC motif and the Mif2-homologous region are both important for centromere localization (Figures S3A–S3C), consistent with the results

obtained with budding yeast and fly CENP-C proteins (Cohen et al., 2008; Heeger et al., 2005). While Cnp3 localization depends on Cnp1, a CENP-A homolog in fission yeast (Figure S3D), our two-hybrid assay using the C-terminal domain of Cnp3 did not detect interaction with the Cnp1 peptide (data not shown) and two-hybrid screening did not reveal any other kinetochore proteins other than a meiosis-specific Moa1 (Figure 2A, also see below). Thus, these results are consistent with the previous suggestion that the C-terminal region may associate directly with centromeric DNA as well as with CENP-A nucleosomes (rather than CENP-A peptide itself) (Ando et al., 2002; Talbert et al., 2004). While the N-terminal domain of CENP-C is dispensable for centromeric localization, this domain is still required for kinetochore function in mitosis (Figure S3E). Therefore, the Pcs1 and Fta1 proteins that interact with the N-terminal domain might be directly related to the kinetochore function of CENP-C.

Centromeric Localization of Pcs1 Depends on Cnp3

The Pcs1 protein was recently characterized as a kinetochore factor required for clamping the kinetochore region to make it compact and facilitating the attachment of the single kinetochore to several microtubules emanating from a single spindle pole; *pcs1Δ* cells frequently produce merotelic attachment and lagging chromosomes at anaphase (Gregan et al., 2007; Rabitsch et al., 2003) (Figure 3A). The observation of Pcs1 tagged with GFP revealed that the majority of Pcs1 accumulates in the nucleolus, while a small portion localizes at centromeres throughout interphase until metaphase. At anaphase, Pcs1 mostly relocates to the spindle and returns to the nucleolus and centromeres at G1 phase (Figure 3B). Importantly, most *cnp3Δ-a* cells specifically lose interphase centromeric Pcs1 signals (Figure 3C), while Cnp3 localization remains intact in *pcs1Δ* cells (Figure S4A), suggesting that Pcs1 functions downstream of Cnp3 at centromeres. This fact fits with the previous observation that lagging chromosomes are frequently generated in *cnp3Δ* cells (Figure 1C). Thus, these results suggest that the
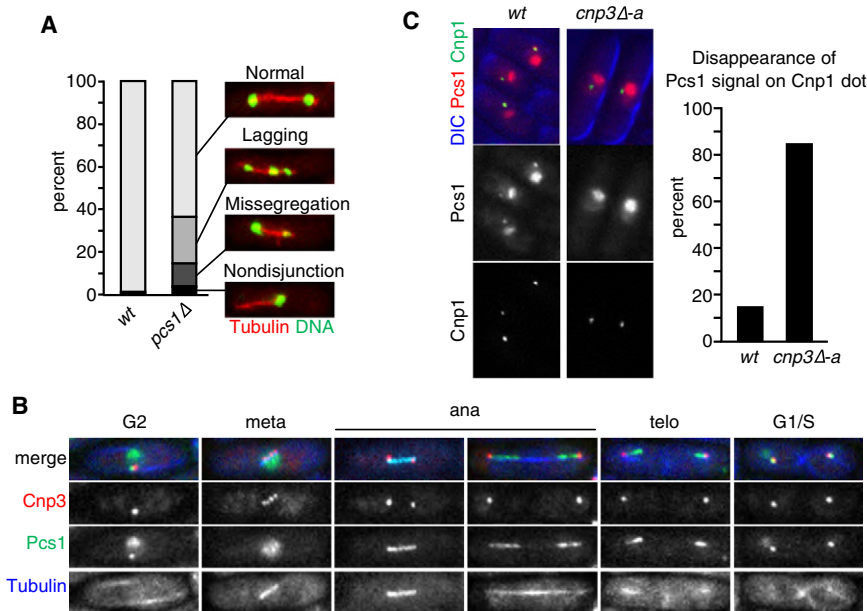
Figure 3. Cnp3 Is Responsible for the Centromeric Localization of Pcs1

(A) Chromosome segregation in wild-type and *pcs1Δ* cells. Cells were grown at 30°C. Chromosome segregation was observed in anaphase cells (n > 100).

(B) Localization of Pcs1 during the cell cycle. Cells expressing *pcs1⁺-GFP*, *cnp3⁺-tdTomato*, and CFP tagged α-tubulin were grown at 26°C and observed under a fluorescence microscope.

(C) Centromeric localization of Pcs1 in wild-type and *cnp3Δ* strains. Cells expressing *pcs1⁺-tdTomato* and *cnp1⁺-GFP* were grown at 30°C in minimal medium (MM). Colocalization of the Pcs1 signal with the Cnp1 dot (centromere) was scored in interphase cells (unseptated with a single Cnp1-GFP dot) (n > 100).

recruitment of the Pcs1 complex to centromeres is an important function of Cnp3 in preventing merotelic attachment of the kinetochore.

Fta1/CENP-L Is a Primary Effecter of Cnp3

Fta1, another protein that interacts with N-terminal Cnp3, is a putative component of the Sim4 complex (vertebrate CENP-H-I complex) (Liu et al., 2005; Okada et al., 2006), which plays an essential role in kinetochore assembly and attachment to microtubules. Consistently, Fta1 shows homology with vertebrate CENP-L, a component required for the localization of the CENP-H-I complex (Okada et al., 2006) (Figure S5A). The gene disruption of *fta1⁺* in a diploid strain (*fta1Δ/+*) and subsequence tetrad analysis revealed that *fta1⁺* is an essential gene (Figure S5B). Tagging chromosomal *fta1⁺* with a GFP at the C terminus made the Fta1 protein temperature sensitive; however, Fta1-GFP is largely functional at 30°C or lower temperatures (Figure S5C), rationalizing the usage of Fta1-GFP for localization analysis under these conditions. Fluorescence microscopy confirmed that Fta1 is a kinetochore protein that colocalizes with Cnp3 (Figure S5D) (Liu et al., 2005).

In genetic crossing, we noticed the synthetic lethal interaction between *fta1-GFP* and *cnp3Δ-a*, suggesting a close functional relationship between these factors. To examine the dependency of Fta1 localization on Cnp3 and override the difficulty in generating a *fta1-GFP cnp3Δ-a* strain, we expressed Fta1-GFP from an exogenous chromosomal locus while preserving the endogenous *fta1⁺* allele intact in *cnp3Δ-a* cells and compared the Fta1-GFP signals with those in *cnp3⁺* cells. The centromeric localization of Fta1-GFP is observable throughout the cell cycle in *cnp3⁺* cells but fluctuates with culmination at late G2 or prophase (Figure 4A). Quantitative fluorescence and chromatin immunoprecipitation (ChIP) assays indicated that Fta1-GFP localization at centromeres is substantially reduced in *cnp3Δ-a* cells; Fta1-GFP signals become nearly undetectable during meiosis (Figures 4A–4C). In reverse experiments, Cnp3 localiza-

tion is intact in the *fta1-ts* mutant (see below, Figure S4B) (hereafter, *fta1-GFP* is denoted as *fta1-ts* only when used at the restrictive temperature). These results suggest that centromeric Fta1 localization is largely, if not entirely, dependent on Cnp3, and that Fta1 acts downstream of Cnp3. At a restrictive temperature, *fta1-ts* cells produce detachment of kinetochores from microtubules and nondisjunction of chromosomes at anaphase (Figure 4D), indicative of defective attachment of kinetochores. Consistent with the fact that Fta1 is included in the Sim4-complex, the centromeric localization of Sim4 decreases in the *fta1-ts* mutant (Figure 4E). These results indicate that Fta1 plays a fundamental role in linking the kinetochore to spindle microtubules, as might be true for other members of the Sim4 complex as well (Jin et al., 2002; Kerres et al., 2006; Pidoux et al., 2003).

The residual centromeric localization of Fta1 in *cnp3Δ* cells indicates that there is a secondary Fta1 localization pathway, which would account for the non-lethality of *cnp3Δ* cells despite of the lethality of *fta1Δ* cells. Remarkably, the overexpression of Fta1, but not Pcs1, largely restored the growth of *cnp3Δ-a* cells with the recovery of proper chromosome segregation (Figures 4F and 4G). Accordingly, the centromeric localization of Sim4 was restored in Fta1-overexpressing *cnp3Δ-a* cells (Figure 4H). Consistent with the requirement of the Sim4 complex for the centromeric deposition of Cnp1/CENP-A (Pidoux et al., 2003), the localization of Cnp1 slightly decreased in the *cnp3Δ-a* mutant and Fta1-overexpression restored it (Figure 4I). We noticed that the centromeric localization Fta1 is somewhat increased in the adapted *cnp3Δ-a* cells as compared to the original poorly growing *cnp3Δ* cells, especially at late G2 phase (Figure 4A, compare them at 30°C), consistent with the notion that impaired Fta1 localization is the reason for the poor growth of *cnp3Δ* cells. Finally, the suppression of *cnp3Δ* by Fta1 overexpression was confirmed in the absence of adaptive mutation (Figures S5E and S5F). Thus, our results strongly suggest that the primary function of Cnp3 is to recruit Fta1 to kinetochores, which plays an essential role in kinetochore-microtubule attachment by localizing the Sim4 complex (Figures 4D and 4E).
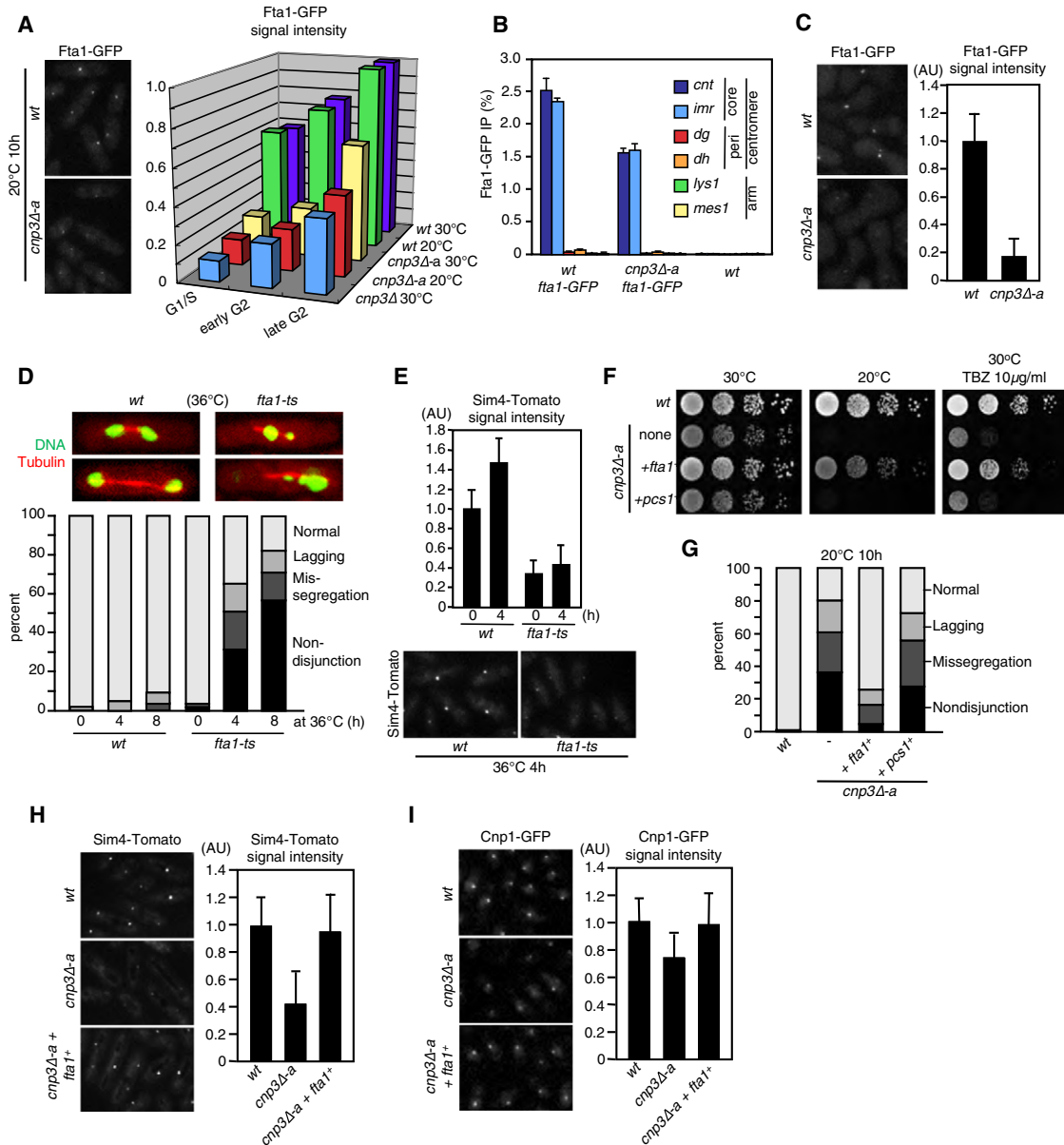
Figure 4. Cnp3 Contributes to Kinetochore-Microtubule Attachment by Recruiting the Fta1-Sim4 Complex onto Centromeres

(A) Fta1 localization in wild-type and *cnp3Δ* cells during mitosis. Cells exogenously expressing *fta1+-GFP* from a chromosome locus were grown at 30°C, then shifted to 20°C for 10 hr. The signal intensity of Fta1-GFP was measured in septated (G1/S), small (early G2), and large (late G2) cells (n = 50).

(B) A ChIP assay with anti-GFP antibodies was used to measure Fta1-GFP levels throughout the indicated chromosome loci in the exponentially growing cells at 30°C. Primer sets for the core centromere (*cnt* and *imr*), peri-centromere (*dg* and *dh*), and arm (*lys1* and *mes1*) on chromosome I were used. Error bars represent the SD (n=3).

(C) Fta1 localization in wild-type and *cnp3Δ* cells during meiosis. Homothallic cells exogenously expressing *fta1+-GFP* were sporulated at 30°C. The signal intensity of Fta1-GFP in meiotic prophase I was measured. Error bars represent the SD (n = 25).

(D) Chromosome segregation in wild-type and *fta1-ts* cells. Cells were grown at 25°C, then shifted to 36°C for 4 hr and 8 hr. Chromosome segregation was observed in anaphase cells (n > 100).

(E) Localization of Sim4 in wild-type and *fta1-ts* cells. *sim4+-tdTomato* tagged strains were grown at 25°C then shifted to 36°C for 4 hr. The signal intensity of Sim4-tdTomato was measured in large (late G2) cells. Error bars represent the SD (n = 50).

(F) Serial dilutions of wild-type, *cnp3Δ-a*, *fta1+* overexpressing *cnp3Δ-a*, and *pcs1+* overexpressing *cnp3Δ-a* cells were grown on YES plates under the indicated conditions.

(G) The indicated cells were grown at 30°C, then shifted to 20°C for 10 hr, and chromosome segregation was observed in anaphase cells (n > 100) as in (D).

(H) Sim4 localization in wild-type, *cnp3Δ*, and *fta1+* overexpressing *cnp3Δ* strains. The indicated *sim4+-tdTomato* cells were grown at 30°C, then shifted to 20°C for 10 hr. The signal intensity of Sim4-tdTomato was measured in large (late G2) cells. Error bars represent the SD (n = 50).

(I) Localization of Cnp1 depends in part on Cnp3. Cnp1 localization in wild-type, *cnp3Δ-a*, and *fta1+* overexpressing *cnp3Δ-a* strains. Cells expressing *cnp1+-GFP* exogenously from a chromosome locus were grown at 30°C, then shifted to 20°C for 10 hr. The signal intensity of Cnp1-GFP was measured in large (late G2) cells. Error bars represent the SD (n = 50).
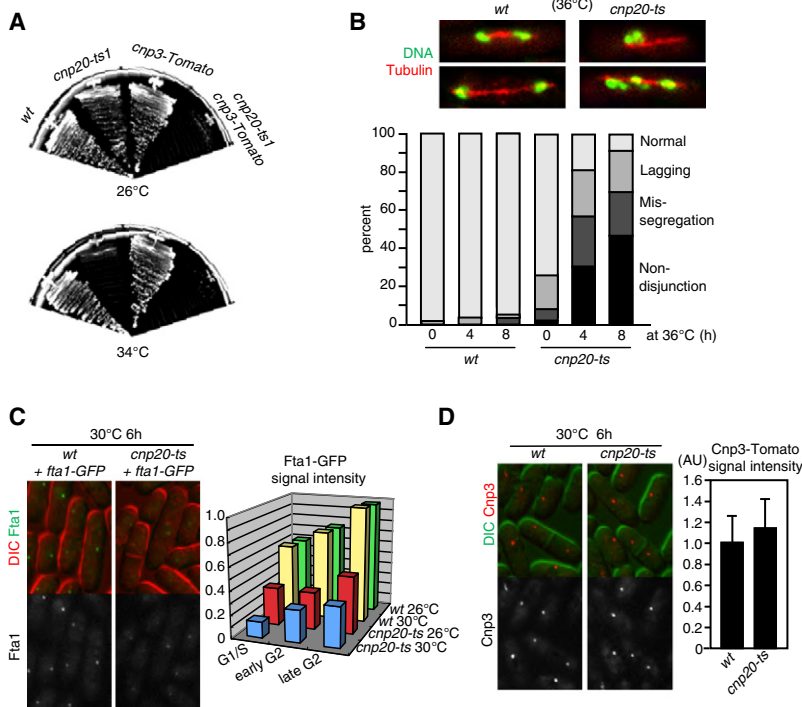
Figure 5. Cnp20, a Putative Homolog of CENP-T, Constitutes the Secondary Fta1 Localization Pathway in Redundant Capacity with Cnp3

(A) Genetic interaction between Cnp3 and Cnp20. Wild-type, *cnp20-ts1*, *cnp3-tdTomato*, and *cnp20-ts1 cnp3-tdTomato* strains were streaked on YES plates, then incubated at 26°C and 34°C. Note that *cnp20-ts1* and *cnp3-tdTomato* show synthetic lethality.

(B) Chromosome segregation in wild-type and *cnp20-ts1* cells. Cells were grown at 26°C, then shifted to 36°C for 4 hr and 8 hr. Chromosome segregation was observed in anaphase cells (n > 100).

(C) Localization of Fta1 depends on Cnp20. Cells expressing *fta1⁺-GFP* exogenously from a chromosome locus were grown at 26°C, then shifted to 30°C for 6 hr. The signal intensity of Fta1-GFP was measured in septated (G1/S), small (early G2), and large (late G2) cells (n = 50).

(D) The localization of Cnp3 does not depend on Cnp20. The signal intensity of Cnp3-tdTomato was measured in late G2 (large cell) in wild-type and *cnp20-ts1* cells grown at 30°C for 6 hr. Error bars represent the SD (n = 50).

Cnp20/CENP-T Promotes the Kinetochore Localization of Fta1 in a Redundant Capacity with Cnp3

It is notable that a recent finding in vertebrates indicates that the localization of the CENP-H-I complex depends on CENP-T/W rather than CENP-C (Hori et al., 2008). We then examined the fission yeast CENP-T homolog Cnp20 (SPBC800.13, Figure S6A). Tagging chromosomal *cnp20⁺* with GFP at the C terminus revealed that Cnp20 is indeed a kinetochore protein that colocalizes with Cnp3 (Figure S6B). Gene disruption of *cnp20⁺* in a diploid strain (*cnp20Δ/+*) and subsequence tetrad analysis revealed that *cnp20⁺* is an essential gene (Figure S6C). By transforming wild-type cells with randomly mutagenized *cnp20⁺* genes, we obtained a temperature-sensitive allele, *cnp20-ts1* (Figure 5A). At a restrictive temperature, *cnp20-ts1* cells undergo detachment of the kinetochores from microtubules and nondisjunction of chromosomes at anaphase, indicative of a defective attachment of kinetochores (Figure 5B). Accordingly, *cnp20-ts1* cells impaired Fta1 localization without diminishing Cnp3 localization (Figures 5C and 5D). Moreover, weak mutant alleles of *cnp3⁺* and *cnp20⁺* show strong synthetic genetic interaction (Figure 5A). These results suggest that Cnp20 constitutes the secondary Fta1 localization pathway in redundant capacity with Cnp3 in fission yeast.

Moa1 Is the Meiotic Effector of Cnp3 Required for Mono-Orientation

The identification of the interaction of Cnp3 with Moa1 also suggests that Cnp3 plays a specific role in the mono-orientation of meiotic kinetochores. Accordingly, *cnp3Δ-a* cells show defects in the centromeric localization of Moa1 at meiosis I (Yokobayashi and Watanabe, 2005) (Figure S7A) along with exhibiting some defects in mono-orientation (Figure 1D and 2:1:1:0 pattern). However, because the defects in canonical kinetochore

functions, such as attachment to microtubules, are predominant in *cnp3Δ-a* cells, it is unclear whether a specific role in mono-orientation can be assigned to Cnp3. To resolve this problem, we sought to isolate *cnp3* mutants that retain normal kinetochore microtubule attachment but fail to recruit Moa1 in meiosis I.

The full-length *cnp3⁺* gene was subjected to random mutagenesis in vitro through a polymerase chain reaction (PCR) using error-prone DNA polymerase. The *cnp3Δ-a* cells, which show cold-sensitive growth and displacement of Moa1 at meiosis I, were then transformed by the mutated *cnp3* fragments to replace the deletion allele. We searched for mutants showing restored canonical kinetochore functions (normal growth at 20°C), but that still failed to localize Moa1 to kinetochores during meiosis I. As a consequence, we obtained 10 mutants named *cnp3-mr* (Moa1-recruitment) (Figure S7). The *cnp3-mr* loci were amplified by PCR and analyzed by direct sequencing in order to determine the mutation site(s). Interestingly, we found that all mutants had at least one mutation within a limited C-terminal region of the Cnp3 protein (aa522-aa623, Figure 6A; Figure S3B), which resides within the Moa1 interaction domain identified by two-hybrid assay (Figure 2D). From them, we selected two point mutants, *cnp3-A552T* and *cnp3-Y607C*, which lose Moa1 signals in meiosis but behave completely like wild-type cells in mitosis; they are intact in the centromeric localization of Fta1, mitotic maintenance of minichromosome Ch16, and growth in the presence of spindle poison (TBZ) (Figures 6B–6D, Figures S7B and S8A). The tagging of endogenous mutant Cnp3 proteins with the tdTomato peptide indicated that the mutant Cnp3 proteins localize normally to centromeres even in meiosis (Figure 6C). Immunoprecipitation experiments indicated that while wild-type Cnp3 proteins coprecipitate Moa1 from meiotic extracts, mutant Cnp3 proteins fail to do so under the same conditions (Figure 6E). These results indicate that the mutant Cnp3 proteins have specifically lost the ability to form complexes with Moa1.

To test the meiotic defects of the *cnp3-A552T* and *cnp3-Y607C* mutants, we monitored meiotic chromosome
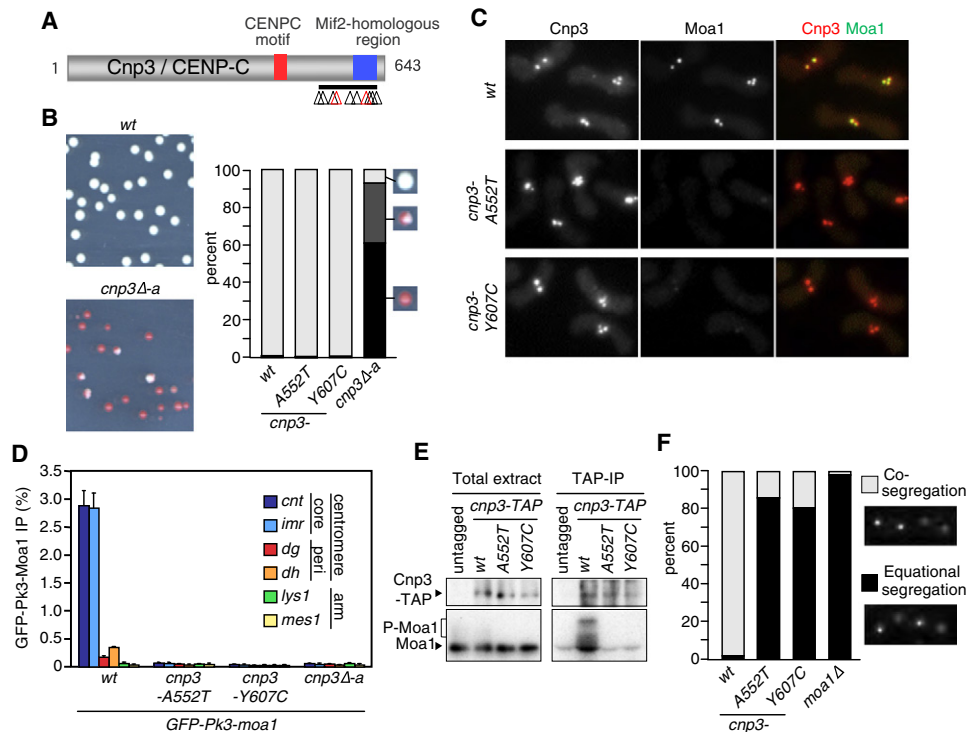
Figure 6. Cnp3 Acts in the Mono-Orientation of Kinetochores by Recruiting Moa1 onto Centromeres

(A) Schematic illustration of the Cnp3 protein. Triangles represent the mutation sites of *cnp3-mr* mutants (also see Figure S3B). Two red triangles correspond to *cnp3-A552T* and *cnp3-Y607C* mutations.

(B) Minichromosome maintenance assay in wild-type, *cnp3Δ-a, cnp3-A552T*, and *cnp3-Y607C* cells. Cells were grown in MM medium lacking adenine at 30°C, then spread onto low adenine YEA plates. The numbers of red, sector, and white colonies were scored (n > 900).

(C) Kinetochore localization of Cnp3-tdTomato and Moa1-GFP was examined in wild-type, *cnp3-A552T*, and *cnp3-Y607C* cells during meiosis. Homothallic cells were induced into meiosis at 30°C and arrested at meiotic prophase I by *mei4Δ*.

(D) Centromere localization of Moa1 in wild-type, *cnp3-A552T*, *cnp3-Y607C*, and *cnp3Δ-a* cells during meiosis. Homothallic cells were induced into meiosis at 30°C and arrested at meiotic prophase I by *mei4Δ*. A ChIP assay was done with anti-Pk antibodies and primer sets described in Figure 4B. Error bars represent the SD (n=3).

(E) Coimmunoprecipitation of Cnp3 and Moa1. Cnp3 was tagged with HA3-TAP in wild-type, *cnp3-A552T*, and *cnp3-Y607C* strains and detected by an anti-HA antibody. Cell extracts were prepared from meiotic cells and precipitated by IgG beads. An untagged wild-type strain was used as a negative control. Moa1 was tagged with GFP-3Pk in all strains and detected by an anti-Pk antibody. Phosphorylated Moa1 (P-Moa1) as well as unphosphorylated Moa1 were coprecipitated with wild-type Cnp3.

(F) *cnp3-A552T* and *cnp3-Y607C* mutants show equational chromosome segregation at meiosis I. The segregation pattern of centromere II in wild-type, *cnp3-A552T*, *cnp3-Y607C*, and *moa1Δ* cells was determined by observing heterozygous *cen2-GFP* signals after the completion of meiosis (n > 100). All strains carry *rec12Δ*.

segregation, expecting that these mutants would mimic *moa1Δ* cells. We marked *cen2*-GFP on only one of the two homologous chromosomes in a zygote and monitored chromosome segregation during meiosis I. In this particular experiment, we used the strains with the *rec12*[+] deletion (the *SPO11* homolog in fission yeast) because the presence of homologous recombination considerably suppresses the mono-orientation defect in meiosis (Yokobayashi and Watanabe, 2005). Simple recombination-defective *rec12Δ* cells show cosegregation of sisters at meiosis I, whereas *moa1Δ rec12Δ* cells display total equational segregation (Yokobayashi and Watanabe, 2005) (Figure 6F). Similarly, *cnp3-A552T* and *cnp3-Y607C* mutant cells, if combined with *rec12Δ*, largely undergo equational segregation at meiosis I (Figure 6F). Thus, we conclude that the *cnp3-A552T* and *cnp3-Y607C* mutants exhibit specific defects in mono-orientation but that kinetochore-microtubule attachment is preserved intact as in *moa1Δ* cells. These results prove the biological significance

of the interaction of Cnp3 with Moa1, which is exclusively required for the localization of Moa1 and the promotion of the mono-orientation of kinetochores at meiosis.

DISCUSSION

CENP-C is a conserved kinetochore component in eukaryotic cells. In all eukaryotes examined so far, CENP-C plays an essential role in cell viability. In fission yeast, however, *cnp3Δ* cells are barely viable despite the fact that most kinetochore proteins are indeed essential in this organism. This indicates that kinetochores that lack CENP-C are partly functional. We identified Fta1/CENP-L, an essential kinetochore protein, as a factor acting downstream of CENP-C, since the localization of Fta1 declines in *cnp3Δ* cells and, crucially, forced localization of Fta1 restores the growth of *cnp3Δ* cells. Although fission yeast *cnp3Δ* cells are not completely lethal, they readily acquire
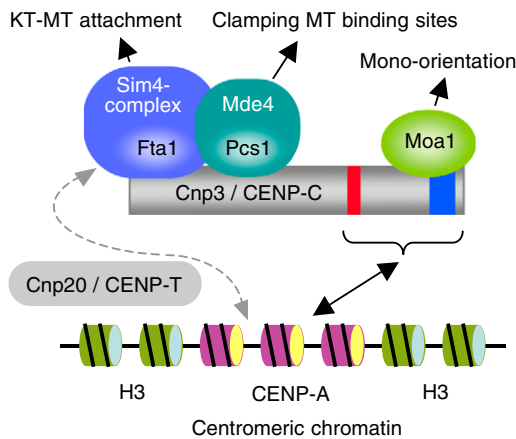
Figure 7. A Schematic Model Illustrating How Cnp3/CENP-C Promotes Faithful Chromosome Segregation in Mitosis and Meiosis

Cnp3/CENP-C acts as a molecular hub for localizing crucial kinetochore proteins or subcomplexes, such as Fta1-Sim4 complex, Pcs1-Mde4 complex and Moa1, and connects them to the centromeric DNA region marked by the CENP-A nucleosome.

extragenic suppressor mutation(s) during proliferation. In this study, we often utilized the stable *cnp3Δ-a* strain, which carry the *pst2-G745R* mutation, although we confirmed the crucial phenotypes, mitotic chromosome segregation defects, and its suppression by Fta1 overexpression by using the primary culture of *cnp3Δ* cells free from suppressor mutation(s) as well. Pst2 was previously identified as a component of the Clr6 histone deacetylase complex II (Nakayama et al., 2003; Nicolas et al., 2007). It is also shown that a mutation in the histone acetyltransferase (HAT) complex gene impairs gene silencing in the core centromeric region as well as the kinetochore function, whereas mutations in the Clr6 histone deacetylase complex II gene have the opposing effects on silencing and kinetochore function (Minoda et al., 2005; Nicolas et al., 2007). Therefore, the balance of histone acetylation at the core centromeres may act to regulate the kinetochore integrity, rationalizing the *pst2* mutation and potential increase in histone acetylation at centromeres strengthened kinetochore assembly. It is, therefore, possible that any mutations in the Clr6 histone deacetylase complex II or other HDAC potentially suppress *cnp3Δ*. Since Fta1 localization is restored in *cnp3Δ-a* cells, this suppression might be mediated by the Cnp20 pathway, which is also important for Fta1 recruitment.

We identified Fta1, a subunit of the Sim4 complex (vertebrate CENP-H-I complex) and essential for kinetochore function as a Cnp3-interacting protein. The centromeric localization of Fta1 and Sim4 depends largely on Cnp3, suggesting that these factors function downstream of Cnp3. Crucially, the forced localization of Fta1 largely restores the *cnp3Δ* defects of kinetochores, suggesting that the primary mitotic function of Cnp3 is the recruitment of Fta1 to centromeres. Given that Fta1 is a putative homolog of vertebrate CENP-L, a component of the CENP-H-I complex (Okada et al., 2006), our results indicate that the functional relationships between CENP-L and the CENP-H-I complex are conserved in fission yeast. However, in vertebrates, the localization of the CENP-H-I complex depends on CENP-

T/W rather than CENP-C (Hori et al., 2008). We demonstrated that the fission yeast CENP-T homolog, Cnp20, is also required for the recruitment of Fta1 to centromeres. In addition, *cnp3+* and *cnp20+* show a strong genetic interaction. These results are consistent with the notion that Cnp3 and Cnp20 share a role in localizing Fta1 to the kinetochore in fission yeast (Figure 7). In contrast, kinetochore localization of Nuf2, a component of Ndc80 complex required for kinetochore-to-microtubule attachment (Nabetani et al., 2001), is rather intact in *cnp3Δ-a, fta1-ts* and *cnp20-ts* cells (Figure S9), suggesting that the Ndc80 complex assembles through a distinct pathway in fission yeast (Liu et al., 2005). A recent report revealed the close proximity of CENP-C, CENP-I and CENP-T within the human kinetochore (Wan et al., 2009), suggesting that mammalian CENP-C is also closely associated with CENP-H-I as in fission yeast. Overall, these findings support the close interaction of the crucial DNA-binding kinetochore proteins CENP-C and CENP-T with the assembly of the CENP-H-I complex.

As another Cnp3-interacting protein, we identified Moa1, a meiosis-specific kinetochore protein exclusively required for the mono-orientation of sister kinetochores at meiosis I. We demonstrated that the centromeric localization of Moa1 is Cnp3 dependent, suggesting that Moa1 functions downstream of Cnp3. Our analysis culminated in identifying *cnp3-mr*, point mutants of *cnp3+* defective in the localization of Moa1 and mono-orientation of kinetochores. These mutations clustered in the C-terminal domain of Cnp3, which overlaps with the domain required for the physical interaction with Moa1. Although the C-terminal domain of Cnp3 (Cnp3-C) is sufficient for the interaction with Moa1 in two-hybrid assay, the expression of Cnp3-C alone does not restore Moa1 localization as well as mo-nopolar attachment in *cnp3Δ* cells, despite sufficient localization of Cnp3-C at the centromeres (Figure S10). This result implies that the N-terminal domain of Cnp3 also contributes to the stable localization of Moa1 at centromeres. We think that this Cnp3 function of the N-terminal domain is not mediated by Fta1, since Moa1 localization is unaffected in *cnp20-ts* cells in which Fta1 localization is significantly diminished (Figure S8B). Although our genetic screening for *cnp3-mr* mutations failed to isolate N-terminal mutations, this might be tenable if they affect the canonical kinetochore functions as well. Nevertheless, since we identified *cnp3-mr* alleles that specifically abolish the localization and function of Moa1, our results unequivocally indicate that the function of mono-orientation is separable from the canonical kinetochore function on the CENP-C protein.

In summary, we determined the molecular role of CENP-C in fission yeast (Figure 7). Our results suggest that CENP-C serves as a scaffold for the recruitment of specific kinetochore factors, which enables the construction of highly integrated mitotic and meiotic kinetochores.

EXPERIMENTAL PROCEDURES

Schizosaccharomyces pombe Strain

All strains used in this study are listed in Table S1. The deletion and tagging of endogenous *cnp3+, fta1+, pcs1+, sim4+, nuf2+, cnp20+,* and *mei4+* by GFP, tdTomato, and HA3-TAP were performed by the PCR-based gene targeting method for *S. pombe* using *ura4+, kanMX6 (kanR), hphMX6 (hygR),* and *natMX6 (natR)* genes as selection markers (Bahler et al., 1998; Sato et al., 2005; Shaner et al., 2004). GFP-3Pk-tagged *moa1+* has been described

(Yokobayashi and Watanabe, 2005). To express *fta1*[+], *fta1*[+]*-GFP, pcs1*[+], *cnp1*[+]*-GFP, CFP-atb2*[+], *mCherry-atb2*[+], *tdTomato-cnp3*[+], and *cnp3*[+]*-CFP* genes from exogenous chromosomal loci, the coding sequence of each gene was subcloned under the promoter *Padh13*, *Padh15*, *Padh21*, and *Padh31* (various mutant versions of the *adh1*[+] promoter constructed by introducing point mutations or deletions; promoter strength *Padh13* > *Padh15* > *Padh21* > *Padh31*) or Prec8 (the *rec8*[+] promoter). The resulting plasmids were linearized and integrated at the locus adjacent to the SPAC26F1.12c gene of chromosome I (designated as the *C* locus), the *zfs1*[+] gene of chromosome II (designated as the *Z* locus), or the *lys1*[+] locus.

Yeast Two-Hybrid Assay
Full-length (1-643aa), N-terminal (1–338aa), and C-terminal (338–643aa) regions of the *cnp3*[+] cDNA were subcloned into the vector pGBKT7 and used as bait for screening. An *S. pombe* cDNA library constructed from meiotic mRNAs on vector pVP16 was cotransformed into the yeast strain AH109 with bait, and positive transformants were selected on SC-trp-leu-his-ade plates. Prey plasmids were extracted from candidate clones and sequenced.

Quantification of Fluorescent Signals
To quantify the centromeric fluorescent signals, in-focus images of Fta1-GFP, Cnp3-tdTomato, Sim4-tdTomato, Cnp1-GFP, Naf2-GFP and GFP-Pk3-Moa1 were taken using AxioVision4.6 imaging software (Carl Zeiss). The average unit among the centromeric signals was measured and the background subtracted.

Isolation of the *cnp20-ts1* Mutant
The endogenous *cnp20*[+] gene was tagged with a Flag using the *kanR* gene as a marker. Random point mutations were introduced into *cnp20*[+] by in vitro PCR using the Flag-tagged *cnp20*[+] genomic DNA as a template. Amplified fragments were transformed into a wild-type strain (JY333) and chromosomal *cnp20*[+] was replaced with the mutagenized *cnp20* DNA. Thereafter, clones that showed temperature-sensitive growth ware selected. One ts allele (*cnp20-ts1*) was used for further study.

ChIP Assay
The procedure was carried out essentially as described previously (Yokobayashi et al., 2003). Anti-GFP polyclonal antibodies (BD Biosciences), anti-histone H3 polyclonal antibodies (Abcam), anti-Cnp1 polyclonal antibodies (Takayama et al., 2008), and anti-Pk monoclonal antibodies (MCA1360; Serotec) were used for immunoprecipitation. DNA prepared from whole-cell extracts or immunoprecipitated fractions was analyzed by quantitative PCR with the ABI PRISM7000 system (Applied Biosystems) using SYBR Premix Ex Taq (TaKaRa). The primers used for PCR were all described previously (Kawashima et al., 2007; Yokobayashi et al., 2003). We included an untagged strain in each experiment to account for nonspecific binding in the ChIP fractions. ChIP data from specific antibodies were normalized using data for the pericentromeric and arm regions (*dg, dh, lys1*, and *mes1*) of histone H3 antibodies.

Isolation of *cnp3-mr* Mutants
Random point mutations were introduced into *cnp3*[+] by PCR amplification with an error-prone Ex Taq DNA polymerase (TaKaRa). Genomic DNA from a strain in which the *ura4*[+] gene was integrated at the 3′ untranslated region of the *cnp3*[+] locus was used as a template. The amplified *cnp3 < < ura4*[+] fragment containing upstream 5′ and downstream 3′ untranslated regions was transformed into homothallic *cnp3Δ GFP-3Pk-moa1*^{+} cells (PV508) and selected on SD plates lacking uracil at 32°C. G418-sensitive transformants (clones that underwent correct replacement of the *cnp3Δ::kanR* locus with the mutagenized *cnp3* gene) were then subjected to a cold-sensitivity test, and mitotically healthy clones were induced to meiosis and examined for GFP-Moa1 localization. About 1000 of the relatively healthier clones out of >2000 Ura[+] transformants were subjected to the cold-sensitivity test. The 809 clones that grew normally at 20°C were then induced to meiosis, and the presence of GFP-Moa1 signals on kinetochores at meiosis I was examined. Finally, 10 candidate clones that grew normally at 20°C but failed to recruit Moa1 to centromeres were selected.

Immunoprecipitation
Immunoprecipitation was performed using protein extracts prepared from *pat1-ts*-induced meiotic cells. Cells with a temperature-sensitive *pat1-114* mutation were arrested first in G1 by nitrogen starvation at 25°C, then shifted to 34°C to induce meiosis in a synchronous fashion. Four hours after the temperature shift, cells accumulated at meiosis I were harvested to prepare cell extracts. The HA3-TAP-tagged Cnp3 protein was precipitated with IgG sepharose (GH Healthcare Bio-Sciences) and the coprecipitation of the GFP-Pk3-tagged Moa1 protein was analyzed by western blotting with anti-HA (3F10; Roche) and anti-Pk (MCA1360; Serotec).

Genome-Wide Sequencing
Equal amounts of genomic DNA prepared from the original *cnp3Δ-a* strain (PR726) and a backcrossed *cnp3Δ-a* strain (PR828) were mixed and used to construct a library using a Genomic DNA Sample Prep Kit (Illumina). Sequencing was done with an Illumina Genome Analyzer II (Illumina). Sequencing data were analyzed with two programs, Maq and Edena, by using the *S. pombe* genomic DNA sequence (Sanger Institute) as a reference.

SUPPLEMENTAL DATA

Supplemental Data include one table and ten figures and can be found with this article online at http://www.cell.com/developmental-cell/supplemental/S1543-5807(09)00341-4.

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