**Fission Yeast CENP-C (Cnp3) Plays a Role in Restricting the Site of CENP-A Accumulation**

Michiko Suma,*1 Teppei Kitagawa,*2 Yukiko Nakase,* Norihiko Nakazawa,† Mitsuhiro Yanagida,‡ and Tomohiro Matsumoto*,3

*Radiation Biology Center, Graduate School of Biostudies, Kyoto University, Yoshida-Konoe cho, Sakyo ku, Kyoto, Japan, 606-8501 and †G0 cell unit, Okinawa Institute of Science and Technology Graduate University, Onna-son, Okinawa, Japan, 904-0495

**ABSTRACT** The centromere is a chromosomal locus where a microtubule attachment site, termed kinetochore, is assembled in mitosis. In most eukaryotes, with the exception of holocentric species, each chromosome contains a single distinct centromere. A chromosome with an additional centromere undergoes successive rounds of anaphase bridge formation and breakage, or triggers a cell cycle arrest imposed by DNA damage and replication checkpoints. We report here a study in *Schizosaccharomyces pombe* to characterize a mutant (*cnp3-1*) in a gene encoding a homolog of mammalian centromere-specific protein, CENP-C. At the restrictive temperature 36°C, the Cnp3-1 mutant protein loses its localization at the centromere. In the *cnp3-1* mutant, the level of the Cnp1 (a homolog of a centromere-specific histone CENP-A) also decreases at the centromere. Interestingly, the *cnp3-1* mutant is prone to promiscuous accumulation of Cnp1 at non-centromeric regions, when Cnp1 is present in excess. Unlike the wild type protein, Cnp3-1 mutant protein is found at the sites of promiscuous accumulation of Cnp1, suggesting that Cnp3-1 may stabilize or promote accumulation of Cnp1 at non-centromeric regions. From these results, we infer the role of Cnp3 in restricting the site of accumulation of Cnp1 and thus to prevent formation of de novo centromeres.

**KEYWORDS** centromere, CENP-A, CENP-C, and fission yeast

The centromere is a chromosomal locus on which a microtubule attachment site, termed kinetochore, is assembled in mitosis. It is therefore an essential component for faithful segregation of chromosomes. A variant of histone H3, CENP-A, is specifically incorporated into centromeric chromatin and plays an important role as the base of the kinetochore assembly. (Black and Cleveland 2011; Quénet and Dalal 2012; Catania and Allshire 2014). Distribution of CENP-A must be strictly regulated so that the centromere is positioned and organized appropriately for each species. Although it is generally accepted that centromeres are defined by a sequence-independent epigenetic manner (Allshire and Karpen 2008; Black and Cleveland 2011; Perpelescu and Fukagawa 2011), the underlying mechanisms to deposit and maintain CENP-A at the right place are still elusive.

In most eukaryotes, with the exception of holocentric species, each chromosome contains a single distinct centromere. It has been reported that a chromosome with an extra centromere (dicentric chromosome) causes deleterious problems such as chromosome breakage (Thrower and Bloom 2001; Thrower et al. 2003; Pobiega and Marcand 2010) and cell cycle arrest (Sato et al. 2012). A dicentric chromosome can also be generated by translocation of a centromere followed by meiotic recombination. To avoid these problems, each chromosome contains a single centromere whose position is maintained through generations. As CENP-A histone is a major component found in centromeric chromatin, distribution of CENP-A fundamentally influences formation and positioning of the centromere.

Recent studies have shown that CENP-A accumulation can be found in non-centromeric chromatin as well. In chicken DT-40 cells, minor accumulation of CENP-A is detected around the native centromeres. When the native centromere is experimentally removed, a neocentromere is occasionally assembled by using the minor accumulation of CENP-A as a seed (Shang et al. 2013). In budding yeast, Cse4, a homolog of mammalian CENP-A, is enriched at promoters that...
contain histone H2A (Hildebrand and Biggins 2016). Psh1 is an E3 ubiquitin ligase (Hewawasam et al. 2010; Ranjitkar et al. 2010) that blocks stable Cse4 incorporation into these promoters by targeting mislocalized Cse4 for degradation (Collins et al. 2004). The ubiquitin system is a conserved pathway for preventing ectopic CENP-A accumulation in other organisms including fission yeast and fly (Gonzalez et al. 2014; Moreno-Moreno et al. 2006).

In fission yeast, overexpression of CENP-A causes the promiscuous incorporation of CENP-A near heterochromatic regions (Choi et al. 2012; Castillo et al. 2013; Gonzalez et al. 2014). Most of these loci with CENP-A accumulation do not transform into a neocentromere. It has been shown that the histone H2A variant (Pht1), a NAP family protein (Ccp1) and a histone chaperone FACT play a role in antagonizing CENP-A loading at non-centromeric regions (Choi et al. 2012; Ogiyama et al. 2013; Dong et al. 2016). These factors thus likely prevent formation of neocentromeres.

Interestingly, sub-telomeric regions in fission yeast can accommodate neocentromeres when the native centromere is disrupted (Ishii et al. 2008). Subsequent study has revealed that stable association of CENP-A chaperon, Scm3, facilitates neocentromere formation (Ogiyama et al. 2013). Nonetheless, neocentromere formation is a very rare event (several in ten thousand cells in fission yeast), suggesting that neocentromere formation is strongly suppressed likely by multiple mechanisms.

Having been interested in mechanisms to maintain appropriate distribution of CENP-A and prevent formation of de novo centromeres, we have taken a genetic approach in fission yeast model system. We assumed that a mutant unable to maintain proper distribution of CENP-A would be sensitive to expression of CENP-A at a high level. A strain ectopically expressing fission yeast CENP-A (Cnp1) tagged with GFP (GFP-Cnp1) from an inducible promoter, nmt1, was mutagenized and survivors were screened for mutants, which became temperature sensitive upon overexpression of GFP-Cnp1. We have previously reported a mutant (rpt3-1) identified through the screen (Kitagawa et al. 2014). Unlike the wild type fission yeast in which the localization of CENP-A is limited to the central region spanning 10–20 kb of the centromere, the rpt3-1 mutation allows spread of CENP-A localization to other regions (several in ten thousand cells in fission yeast), suggesting that neocentromere formation is strongly suppressed likely by multiple mechanisms.
at non-centromeric regions. We thus propose a role of the wild type Cnp3 in restricting the site of accumulation of Cnp1 to prevent formation of de novo centromeres.

### MATERIALS AND METHODS

#### Strain, culture media and strain construction

An *S. pombe* haploid wild-type strain Sp6 (h- *leu1-32*) and its derivatives used in this study are listed in Table 1. The culture media were complete YES, minimal EMM2, and sporulation medium MEA and SPA (Moreno et al. 1991). Transformation was done using the lithium method (Ito et al. 1983). The cell number was measured using a hematology analyzer (Sysmex F-520P and Beckman multizer 3 coulter counter).

For tagging GFP to the N-terminal of Cnp1, the *cnp1*<sup>+</sup> gene was PCR-amplified with the primers, Cnp1-F and Cnp-R (Table 3), and cloned into pREP41-GFP(N-end tagging) after digestion with restriction enzymes, Sal I and Not I. The resulting plasmid was used for expression of tagged Cnp1 in restricting the site of accumulation of Cnp1 to prevent the formation of de novo centromeres.

#### Western blotting

Cell extracts were prepared from exponentially growing *S. pombe* cells. The cells were suspended in lysis buffer (25 mM HEPES-KOH at pH 7.5, containing 200 mM NaCl, 10% glycerol, 0.2% NP-40 and protease inhibitors (Nacalai Tesque), 1 mM PMSF, and 1% Trasylol) were disrupted by Beads Smash 12 (WAKENYAKU) at 4°C. After centrifugation the supernatants were used for SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The extracts were separated by SDS-PAGE gel and blotted to nitrocellulose membranes (Funakoshi). Antibodies for western blotting were diluted as follows: mouse anti-GFP (Roche) 1:1000, anti-HA (12CA5, Roche) 1:3000; and TAT1 anti-α-tubulin (gift from K. Gull, Oxford, UK) 1:5000. Blots were developed using ECL reagents, Substrate Plus (Pierce) and ECL prime (GE Healthcare). The intensity of each band was measured by Image J and indicated arbitrarily.

#### Microscopy

*S. pombe* strains were grown in YES or EMM2 medium. Images were acquired on a KEYENCE (BZ-9000) and Delta Vision (Applied Precision LLC, Issaquah, WA, USA). CH350L CCD camera (Photomettric, Tucson, AZ, USA). FISH analyses was performed as described (Funabiki et al. 1993).

### Preparation of digoxigenin-labeled probe DNA

pRA140 (Chikashige et al. 1989) was used to visualize the centromere. The DNA fragments used to prepare the probes were digested by a mixture of restriction enzymes, AluI, *DdeI*, *HaeIII*, *RsaI*, and Sau3AI to yield an average fragment size of 300 bp. These fragments were labeled by digoxigenin-dUTP using the random priming labeling kit (Takara 6045 random primer DNA labeling kit ver.2). Nonreacted nucleotides were removed by microspin G-25 column (GE healthcare).

### Mini-chromosome stability assay

The stability of mini-chromosome was determined by a method developed by (Allshire et al. 1995). First, the cells were plated on YE (for Table 2) or EMM containing 10 μg/ml adenine to determine the initial percentage of the cells carrying the mini-chromosome. After incubation in the non-selective medium (Ade<sup>−</sup>), the final percentage of the cells carrying the mini-chromosome was determined. The rate of mini-chromosome loss per division was estimated using the formula: loss rate = 1-[(F/I)<sup>1/N</sup> (F, the final percentage of cells carrying mini-chromosome; I, the initial percentage of cells carrying mini-chromosome; N, the number of generations between F and I)].

### Chromatin immunoprecipitation (ChIP) assay

ChiP was performed as described previously (Kitagawa et al. 2014).

### Isolation of the *cnp3-1* mutant

A strain (TK5: h* leu1-32::nmt1-GFP-cnp1*<sup>+</sup> -leu1<sup>+</sup>) which was able to express fission yeast CENP-A. Cnp1, tagged with GFP from the nmt1 promoter, was chemically mutagenized as described (Matsumoto and Beach 1991). Exponentially growing cells suspended in TM buffer (50 mM Tris malate (pH 6.0) were treated with NTG (500 μg/ml) at 26°C for 20 min and washed three times with TM buffer. Cells were grown in the YES liquid medium for 4h at 26°C and washed with EMM2 medium containing thiamine. Cells were grown in the same medium thiamine and grown at 26°C. The survivors were plated on the EMM2 plates containing thiamine and grown at 26°C. They were subsequently
transferred onto EMM2 plates with and without thiamine by replica and grown at 36°C. The strains that exhibited temperature sensitivity only when GFP-Cnp1 was expressed were selected. They were then individually examined under a fluorescence microscope. We scored mutants with abnormal distribution of GFP-Cnp1 in the nucleus. Tetrad analysis indicated that five strains including the cnp3-1 mutant carried a single mutation responsible for the phenotypes (temperature sensitivity and abnormal distribution of GFP-Cnp1).

**Isolation of the cnp3+ gene**

By using the temperature sensitivity as a selection marker, a genomic DNA fragment complementing the temperature sensitivity was isolated.
from a fission yeast genomic DNA library. The integration mapping proved that this fragment originated from the cnp3-1 locus. A PCR-based strategy was used to identify the mutation site within the cnp3+ coding sequence. The cnp3-1 mutant gene contained mutation of S508.

**Data availability**

All strains and plasmids are available upon request. All data necessary for evaluating our conclusions are represented within this paper. Supplemental material available at Figshare: https://doi.org/10.25387/g3.6618920.

**RESULTS**

**Isolation of cnp3-1**

A strain (TK5: h- leu1-32:nmt1-GFP-cnp1+ -leu1+) which was able to express fission yeast CENP-A, Cnp1, tagged with GFP from the nmt1 promoter was chemically mutagenized and survivors were screened for mutants, which became temperature sensitive upon overexpression of GFP-Cnp1. In this study, we characterized one of the mutants identified through the screen.

Genetic analysis and subsequent gene cloning revealed the temperature sensitivity upon overexpression of Cnp1 to be attributable to a mutation on the cnp3+ gene encoding a fission yeast homolog of CENP-C. The mutant is thus designated cnp3-1 hereafter. Sequencing of the cnp3-1 mutant gene identified a single point mutation changing TCT of the 508th codon to TTT, resulting in the change in the amino acid from serine to phenylalanine (Figure 1A). As shown in Figure 1B, the cnp3-1 mutant becomes temperature-sensitive upon overexpression of Cnp1, but not histone H3, demonstrating the specific sensitivity of the cnp3-1 mutant to Cnp1.

**GFP-Cnp1 foci in the cnp3-1 mutant**

In order to examine distribution of ectopically expressed GFP-Cnp1 in the nucleus, we observed fission yeast cells overexpressing GFP-Cnp1 microscopically in various genetic background (Figure 1C and Figure 2A). We also examined the level of GFP-Cnp1 by immunoblot with an antibody to GFP. As shown in Figure 1D, GFP-Cnp1
was expressed in the cnp3-1 mutant at a level similar to that in the wild type strain at 26°C and 36°C. Likewise, it was expressed at comparable levels in the three strains (wild type, cnp3-1 mutant and deletion strain for the cnp3+ gene) at 30°C and 36°C (Figure 2B).

Because three centromeres of fission yeast cluster adjacent to the spindle pole body, SPB (Funabiki et al. 1993), the fluorescent signal of GFP-Cnp1 expressed from the native promoter appears as a discrete single speckle in each cell. Microscopic observation revealed that the signal of GFP-Cnp1 expressed from the native promoter appeared as a single speckle, while the intensity of the fluorescent signal was reduced, the level of Cnp3-1 mutant protein at the centromere significantly decreased, and chromosomes segregated unequally in 20% of the cells (Figure 3A and B).

We next attempted to investigate the cnp3-1 mutant under physiological conditions. The experiments described below were performed with cells expressing Cnp1 from the native promoter, unless otherwise stated. As described previously, tagging GFP to the N-terminal of Cnp1 does not affect growth of fission yeast (Takayama et al., 2008). To visualize the Cnp3 protein, mCherry was tagged to the C-terminus of Cnp3 and Cnp3-1 mutant protein, respectively. When Cnp1 is solely expressed from its native promoter, the cnp3-1 mutant is able to grow at 36°C. Microscopic observation of the mutant at 36°C, however, revealed that 1) although the signal from GFP-Cnp1 expressed from the native promoter appeared as a single speckle, the intensity of the fluorescent signal was reduced, 2) the level of Cnp3-1 mutant protein at the centromere significantly decreased, and 3) chromosomes segregated unequally in 20% of the cells (Figure 3A and B).

GFP was tagged to Cnp3 and Cnp3-1, respectively, and examined by immunoblot as well as ChIP (Figure 4A and C). As shown in Figure 4B and C, the level of Cnp3-1 mutant protein slightly reduced at 36°C. ChIP analysis indicated that the amount of Cnp3-1

Figure 3 Characterization of the cnp3-1 mutant. (A) The strains expressing GFP-tagged Cnp1 and mCherry-tagged Cnp3 (WT, MS548), Cnp3-1 mutant protein (cnp3-1, MS549) from the native promoter were grown at 26°C or 36°C for 24 hr and observed under a fluorescence microscope. The white arrow heads indicate mis-segregated chromatin mass scored as abnormal in (B). The bar indicates 10µm. (B) The statistic analysis of (A).
bound to centromere chromatin decreased upon the shift to the restrictive temperature (Figure 4A and B). ChIP analysis for Cnp1 also indicated that the amount of Cnp1 bound to centromere chromatin significantly decreased in the cnp3-1 mutant at 36°C (Figure 5).

The centromere binding activity was mapped at a domain of Cnp3 spanning from 414 to 643 amino acids (Tanaka et al. 2009). This domain was fused with GFP, designated F(414-643)-GFP, and expressed in the wild type strain. As shown in Figure 6, F(414-643)-GFP was colocalized with SPB, a marker for the cluster of the three centromeres. When the mutation of cnp3-1 (S508F) was introduced in the domain, designated F(414-643 S508F)-GFP, it was colocalized with SPB at 26°C, but not at 36°C, indicating that F(414-643 S508F)-GFP could recapture the phenotype of the full length Cnp3-1 mutant protein.

These results suggested that the Cnp3-1 mutant protein lost its binding activity to the centromere and allowed a partial loss of Cnp1 from the centromere at 36°C, and prompted us to examine the function of the centromere in the mutant. As shown in Table 2, the stability of the mini-chromosome Ch16 was reduced in the cnp3-1 mutant, indicating a low activity of the centromere in the mutant.

Ectopic foci of Cnp1 in cnp3-1
As described above, the cnp3-1 mutant was prone to promiscuous accumulation of Cnp1 at 36°C when Cnp1 was overexpressed. We speculated that when Cnp1 was present in excess, Cnp3-1 mutant proteins might interact with Cnp1 and promote promiscuous accumulation of Cnp1. In order to test this scenario under a physiological condition, we attempted to increase the level of free Cnp1 by disabling a system to incorporate Cnp1 into centromeric chromatin. It has been shown previously that Mis18 is responsible for incorporation of Cnp1 into centromeric chromatin (Hayashi et al. 2004; Williams et al. 2009). As shown in Figure 7A, the intensity of GFP-Cnp1 foci decreased at 36°C in the temperature sensitive mis18-818 mutant. Remarkably, in the double mutant (mis18-818 cnp3-1), multiple foci of GFP-Cnp1, which were brighter than those in the single mis18-818 mutant, were
observed (Figure 7). We speculated that foci of GFP-Cnp1 were formed ectopically in the cnp3-1 mis18-818 double mutant for the following three reasons; 1) ChIP analysis (Figure 5) indicated that the level of Cnp1 bound to the native centromere was extremely low, 2) the foci were formed even in the mis18-818 mutant defective in CENP-A deposition in the native centromere, and 3) the number of the foci in a nuclei occasionally exceeded three, the number of the fission yeast centromeres (Figure 7A).

We next examined distribution of Mis12, a protein localized at centromeres in a Cnp1-independent manner (Goshima 2003). As shown in Figure S1, S2 and S3, Mis12 was observed as a discrete single speckle in the cnp3-1 mis18-818 and cnp3-1 mid18-262 double mutant at 26°C (Figure S1) and 36°C (Figure S2 and S3), suggesting that the centromere cluster was normally maintained. FISH analysis with a probe for the centromere also indicated that the centromere cluster was normally maintained (Figure S4). Furthermore, multiple foci of GFP-Cnp1 formed at 36°C were found at positions different from the speckles of Mis12 (Figure S2), demonstrating that the foci of GFP-Cnp1 in the cnp3-1 mis18-818 double mutant were ectopic.

As shown in Figure S5 and S6, multiple foci were also observed when the cnp3-1 mutation was combined with mis6-302 or mis16-53, a mutation defective in deposition of Cnp1 into centromeric chromatin.

Finally, we examined direct contribution of Cnp3-1 mutant protein to ectopic formation of Cnp1 foci by localizing Cnp3-1 proteins. In the cnp3-1 mutant, weak fluorescent signal from Cnp3-1-mCherry was colocalized with GFP-Cnp1, which presumably represented the native centromere, indicating that the Cnp3-1 mutant protein lost the activity to bind to the native centromere (Figure 8A). In the double mutant (mis18-818 cnp3-1), the signal from Cnp3-1-mCherry, which was easily discernable, was colocalized with multiple GFP-Cnp1 foci, which were likely ectopic. As shown in Figure 8B, more than 33% of the double mutant cells exhibited multiple foci of GFP-Cnp1.

Based on the observation, we speculated that Cnp3-1 mutant protein preferentially recognized ectopically accumulating GFP-Cnp1 and might promote further accumulation.

**DISCUSSION**

In this study we have characterized the mutant of cnp3+ gene (cnp3-1), which encodes a fission yeast homolog of mammalian CENP-C, with and without overexpression of Cnp1. The mutation site was mapped at S508, which is within the DNA binding domain of Cnp3 (Tanaka et al. 2009).

When Cnp1 is not overexpressed, the cnp3-1 mutant is viable from 26°C to 36°C. Both microscopic observation and ChIP analysis have revealed that the Cnp3-1 mutant protein looses the binding activity to the native centromeres at 36°C. In addition, the level of Cnp1 bound to centromeres decreased. It has been shown by an in vitro study that human CENP-C stabilizes CENP-A nucleosomes on chromatin through physical interaction (Falk et al. 2015). We thus speculate that the reduction in the level of Cnp1 on centromeres is a direct consequence of a loss of the Cnp3-1 mutant protein from centromeric chromatin.

When Cnp1 is overexpressed, the cnp3-1 mutant is unable to grow at 36°C. Microscopic observation revealed that multiple foci of
GFP-Cnp1 were assembled in the mutant. Because we found cells (32%) with more than three foci of GFP-Cnp1 (Figure 2C), some of the multiple foci, at least, are ectopically assembled. In a vast majority of the cnp3Δ strain overexpressing Cnp1, no focus of GFP-Cnp1 was observed, indicating that loss of function of the cnp3+ gene does not result in promiscuous accumulation of Cnp1. We therefore speculate that the single amino acid change, S508F, in Cnp3 causes a gain of function mutation.

The Mis18 protein forms a complex with Mis16 and is required for centromere localization of Scm3, a Cnp1-chaperone (Hayashi et al. 2004; Williams et al. 2009). Consistently with the function of Mis18, incorporation of GFP-Cnp1 into centromeric chromatin was significantly reduced in a temperature sensitive mutant, mis18-818. Interestingly, when the mis18-818 mutation was combined with the cnp3-1 mutation, which is also defective in incorporation of Cnp1 into the native centromeres, multiple Cnp1-foci were observed. As Mis12, a protein localized at the centromere independently from Cnp1, appeared as a single speck, the cluster of the native centromeres is normally maintained in the double mutant, mis18 cnp3-1. Multiple Cnp1-foci therefore are ectopic. Furthermore, the Cnp3-1 mutant protein is associated with these foci, suggesting that it may contribute to ectopic accumulation of Cnp1.

It has been demonstrated that human CENP-A is overexpressed in some aggressive cancer cells, where it can be promiscuously incorporated in non-centromeric regions in the form of heterotypic nucleosomes containing H3.3 (Lacoste et al. 2014). The heterotypic nucleosomes is as stable as the CENP-A homo-nucleosome and can bind to CENP-C in vitro (Arimura et al. 2014). Furthermore, Cnp1 accumulation normally depends on Cnp3 (Tanaka et al., 2009). Taking these reports and our results together into consideration, we speculate that 1) the Cnp3-1 mutant has lost the specific binding activity to the native centromere, where the CENP-A homo-nucleosome is a major
component in chromatin and 2) it binds to the heterotypic nucleosomes containing H3.3 more efficiently than the wild type Cnp3 protein, and finally 3) when Cnp1 is present in excess (in cells overexpressing Cnp1, or in the double mutant, mis18 cnp3-1, where deposition of Cnp1 into the native centromeres is prevented), the Cnp3-1 protein preferentially binds to and stabilizes the heterotypic nucleosomes promiscuously accumulated on non-centromeric regions. Alternatively it is possible that the Cnp3-1 protein may stabilize the CENP-A homo-nucleosomes promiscuously deposited at non-centromeric regions.

With the specific binding activity to the native centromere, Cnp3 serves as a part of the epigenetic mechanism to restrict the position of CENP-A accumulation and prevents formation of de novo centromeres.

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Figure 8 Colocalization of Cnp1-foci and Cnp3-1 in mis18 cnp3-1. (A) Both Cnp1 tagged with GFP and Cnp3 (or Cnp3-1) tagged with mCherry were expressed from their native promoters in the four strains (WT: MS548, cnp3-1: MS549, mis18-818: MS546 and cnp3-1 mis18-818: MS545). They were grown as in Fig. 7A. The white arrows indicate the Cnp3 (Cnp3-1)-foci. The bar indicates 10 μm. (B) The statistic analysis of (A).
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