Plasticity and Epigenetic Inheritance of Centromere-specific Histone H3 (CENP-A)-containing Nucleosome Positioning in the Fission Yeast*

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Background: CENP-A nucleosomes support kinetochore assembly.

Results: Cnp1/CENP-A occupancy, indicated by silencing of the reporter gene, varies among genetically homogenous cells.

Conclusion: Centromeric chromatin organization is flexible and inherited epigenetically.

Significance: How CENP-A nucleosomes are inherited is potentially important for assembling one functional centromere per chromosome and may serve as an experimental system to elucidate the general mechanisms for epigenetic inheritance.

Although the centromere is a designated region on each chromosome, usually spanning tens of kilobases up to several megabases in many eukaryote organisms, the underlying DNA sequence in general is neither necessary nor sufficient to specify centromere identity. Noticeable exceptions include the nematode Caenorhabditis elegans, in which the microtubule binding sites are distributed throughout the chromosome instead of being restricted at a discrete single locus (2), and several closely related fungal species represented by Saccharomyces cerevisiae, in which a specific 125-bp DNA segment is sufficient to direct centromere and kinetochore assembly (3, 4). In most cases, epigenetic markers (in particular, nucleosomes containing a unique histone H3 variant, CENP-A (or CenH3)) play a pivotal role in determining the location of centromere on each chromosome. In all eukaryotic organisms, including the budding yeast S. cerevisiae, CENP-A nucleosomes are found exclusively in all active centromeres and are essential for kinetochore assembly and functions (5–10). Accumulating evidence has begun to illuminate the unique structural properties of CENP-A nucleosomes that enable them to be recognized as the centromeric markers (11–15). Specific kinetochore proteins have been identified (CENP-C, CENP-N in human cells) that bind directly to CENP-A nucleosomes and are postulated to act together with the CENP-A nucleosomes as the physical foundation for kinetochore assembly (16, 17). Thus, understanding how the sites of CENP-A incorporation are determined would in essence provide the answer to how the centromere is specified on each chromosome. Furthermore, precise information about CENP-A nucleosome positioning would be useful for delineating the architecture of the kinetochore (see below).

Two distinct CENP-A deposition processes are implicated in the establishment and the maintenance of a centromere (18). One is de novo CENP-A deposition on chromatin without pre-existing CENP-A, which occurs on artificial chromosomes (19, 20) and possibly on native chromosomes that occasionally lose the incorporated CENP-A. The alternative, which is more com-

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monly utilized during vegetative growth, is the replenishment of CENP-A after DNA replication. Upon replication of the genome, preexisting CENP-A nucleosomes are inherited and partitioned between the replicated sister chromatids. Nascent CENP-A molecules are subsequently deposited to replenish the diluted CENP-A on each chromatid. De novo CENP-A (Cnp1) deposition in fission yeast requires the pericentromeric heterochromatin regions, whereas the propagation of Cnp1 chromatin does not (20), indicating mechanistic differences between these two processes. Interestingly, there is marked variation among different organisms in the timing of CENP-A replenishment (e.g. during S phase in budding yeast (21), in anaphase in Drosophila early embryonic cells (22), and in the subsequent G1 phase in mammalian cells (23)). Two independent investigations have led to somewhat different conclusions for CENP-A/Cnp1 deposition in fission yeast. One suggests that two redundant pathways operate in S or G2 phase, respectively (24), whereas a recent study concludes that Cnp1 is replenished exclusively in G2 phase (25). Overall, a common feature seen in various species is that CENP-A replenishment in many cases may occur without a tight junction with DNA replication (26). An increasing number of factors have been implicated in CENP-A deposition and/or maintenance (reviewed in Ref. 27). Cells deficient in one of these proteins commonly have a diminished level of CENP-A at centromeres. The role of each protein and their coalescence in the establishment and/or maintenance of CENP-A positioning await future studies.

Much less is known regarding the spatial organization of CENP-A at the centromere, the mechanism for its establishment, and whether it is maintained throughout cell generations. Although CENP-A nucleosomes are found exclusively in centromeres, canonical H3 nucleosomes are also found in multiple organisms and carry a distinct covalent modification (histone H3 Lys-4 methylation) in fission yeast (28). In fly and human cells, patches of CENP-A nucleosomes are interspersed with patches of canonical histone H3 nucleosomes on a stretched centromeric chromatin. This distinct pattern of CENP-A nucleosome positioning suggests that it may play a role in influencing higher order folding of centromeric chromatin and, furthermore, kinetochore geometry (9, 29, 30). Specifically, to initiate kinetochore assembly only on the side of the chromosome facing the spindle pole, the centromeric chromatin fiber needs to fold to bring linearly dispersed CENP-A patches together into a single compact region on the chromosome surface. Elaborate models have been proposed for the folding patterns of the chromatin fiber, such as the “amphipathic superhelix” (30) and the “layered boustrrophodon” arrangement (29). Despite its potential importance for centromere and kinetochore assembly, detailed information about CENP-A nucleosome positioning is lacking. The underlying centromeric DNAs in many organisms, including humans and flies (reviewed in Ref. 12), consist of very large DNA segments (hundreds of kilobases up to several megabases) of highly repetitive sequences, making it difficult to determine the sizes and precise positions of the CENP-A patches.

The fission yeast Schizosaccharomyces pombe is a suitable model for delineating CENP-A/Cnp1 nucleosome positioning in a regional centromere. In addition to its long history of usage for cell division and chromatin studies, several features of the fission yeast centromere make it particularly suitable for this study. Centromeres in fission yeast encompass a significant section of the chromatin (ranging from 40 to 100 kb), representing the common type of regional centromere. Yet, it is experimentally trackable in comparison with the much larger centromeres in other organisms (as large as megabases). The DNA sequences of fission yeast centromeres are known, and their functional domain organization is well defined (31). Each centromere consists of a central core domain, flanked by heterochromatic outer repeat domains. The central core region, directly occupied by Cnp1 and the kinetochore components, is composed largely of unique DNA sequences. Interestingly, reporter genes inserted in the central core are silenced stochastically, exhibiting the typical positional effect variegation (PEV)2 phenomenon (32). Although the mechanism of PEV in centromeres is not understood, the fact that variegation in gene expression occurs in the central core suggests that certain properties of the centromeric chromatin are regulated epigenetically.

We previously determined the nucleosome positioning in the central core using tiling microarrays and found that nucleosomes irrespective of their histone composition are positioned in an orderly array, with a regular interval between each other (33). Multiple lines of investigation, using quantitative fluorescence microscopy, have shown that on average, over 50% of total nucleosomes in centromeres contain Cnp1 (25, 34), although a separate study showed a much higher number of Cnp1 molecules residing in centromeres, exceeding the full capacity of centromeric chromatin (35) (see Fig. 2 for details).

We sought to determine the positioning of Cnp1 nucleosomes with the centromeric core. Here, we present evidence indicating that within a cell population, Cnp1 nucleosomes are flexibly positioned throughout the entire central core region within a cell population and that Cnp1 occupancy directly correlates with the silencing of the underlying reporter genes. We developed a pedigree analysis assay to track the reporter gene expression states at the individual cell level through multiple cell divisions. By this assay, we show that the positions of Cnp1 nucleosomes are mostly inherited epigenetically throughout cell generations but also undergo repositioning at a marked rate per generation.

**EXPERIMENTAL PROCEDURES**

**Yeast Media and Culture Conditions**—Yeast media and growth conditions were essentially as described (36). To visualize ade6 gene ON/OFF, YE/4S (or uracil dropout low adenine) media were used: Edinburgh minimal medium containing leucine, histidine, and lysine at 250 mg/liter each with adenine at 10 mg/liter. Also, to select for ura4 gene expression, 5-fluoroorotic acid (5FOA; U. S. Biological Co.) was added to medium at 1 g/liter. All strains were grown at 25 °C. The S. pombe strains used in this study are listed in Table 1.

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2 The abbreviations used are: PEV, positional effect variegation; 5FOA, 5-fluoroorotic acid; WCE, whole cell extract; qPCR, real-time quantitative PCR.
Chromatin Immunoprecipitation and Nucleosome Immunoprecipitation—ChIP was performed as described (37). To grow the cultures of the “tandem” strain for Cnp1 nucleosome immunoprecipitation, cells were first grown on EMM/3S/low ade plates and then picked and pooled and further amplified in 1,000 ml of YE/4S/5FOA medium for 12 h at 25 °C. To select for both ura4/ade6 on, colonies that were predominantly white on EMM/3S/low ade plates were picked and pooled and further amplified in 1,000 ml of YE/4S/5FOA plates that were predominantly red were picked and further amplified in 1,000 ml of EMM/3S/low ade liquid medium for 12 h at 25 °C. 

To select for both ura4+ and ade6+, colonies that amplify the “red” or “white” cells (at least 4–5 days, allowing colony formation and color developing on colony surface. Each Petri dish (10 cm in diameter) usually accommodates 10 pedigrees. 

Cell division numbers are counted as followed (see Fig. 7A for illustration). Generation I has one cell division, generation II has two cell divisions, and generation III has four cell divisions. Adding these divisions, the total number of cell divisions that lead to an eight-cell pedigree is seven. 

To score the switching events, the ancestor cell of each pedigree is first judged to be ade6+.ON or ade6+.OFF based on the color of the majority of the pedigree. This classifies the cell divisions into two groups, those amplify the “red” cells and those amplify the “white” cells. Judgment on the ancestor cells further allows the determination of the nature of a switching event (red to white or the reverse) and the stage at which it is most likely to occur. For example, pedigree 1 in Fig. 7A fits the interpretation that a red-to-white switch occurred in a cell division in generation II that produced the mother cell of colonies 3 and 4. On the rare occasions (about 5% of all pedigrees) that four red and four white colonies are found in an eight-colony pedigree, a discretionary decision is made based on the majority of the founding cells in the same experiment. The switching rates are calculated by dividing the total numbers of cell divisions that amplify the “red” or “white” cells (>500 for each experiment) by the numbers of the red-to-white or white-to-red switching events, respectively.
RESULTS

Mapping Cnp1 Nucleosomes by Anti-Cnp1 Nucleosome Immunoprecipitation/High Throughput Sequencing—We have previously demonstrated that the centromeric core is composed of an orderly array of nucleosomes. Specifically, for the cnt2 region, 27 nucleosome positions without distinction of the nucleosome types were identified (33). To determine which positions are occupied by the Cnp1 nucleosomes, we performed immunoprecipitation of nucleosomes derived from micrococcal nuclease digestion of yeast chromatin, followed by high throughpt sequencing of co-purified mononucleosomal DNA (Cnp1 nucleosomal DNA). In parallel, mononucleosomal DNA was also purified from the micrococcal nuclease digestion mixture prior to affinity purification (total nucleosomal DNA) and subjected to high throughput sequencing. The position and the relative abundance of each nucleosome were deduced by mapping sequenced DNA reads to the reference genome. The positions of total nucleosomes in the centromeric cores determined by high throughput sequencing (green line in Fig. 1) are highly similar to the results of our previous study using tiling microarray (33). All Cnp1 nucleosomal peaks (red line in Fig. 1) were identified within the centromeres, confirming the specificity of the immunoprecipitation. Cnp1 nucleosomes were identified in nearly all nucleosome positions within the central core.

Several independent studies, including an earlier work of ours, have measured the Cnp1 molecule copy number within the centromeres of fission yeast, using GFP-based quantitative fluorescence microscopy, but have reached inconsistent con-
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A. GFP intensity of the centromere cluster in G2/M phase nucleus was quantified by fluorescent microscopy in various versions of the Cnp1-GFP (S65T) construct (see “Experimental Procedures” for details of strain construction). Imaging was performed at 25 °C. For each sample, n = 30; the error bar represents 3D.8, impact of temperature on the GFP signal intensity measured in various Cnp1-GFP strains. Two independently constructed, but identical in design, Cnp1-GFP (S65T) strains and one Cnp1-mEGFP strain were compared at 25 and 32 °C. P values were calculated by t test. a.u., absorbance units.

B. GFP(S65T) molecules/kinetochore or a 112 molecule/kinetochore cluster in G2/M phase, equivalent to ~13 Cnp1 nucleosomes/centromere. A separate study, however, showed a much higher number of Cnp1 molecules residing in centromeres, exceeding the full capacity of centromeric chromatin (35). The reason underlying the discrepancy is unclear, although our reexamination of the same yeast strains used in the study of Coffman et al. (35) confirmed the partial coverage of centromere by Cnp1 nucleosomes. Furthermore, we verified that increased expression of Cnp1 leads to enhanced incorporation of Cnp1 in centromeres, supporting the notion that endogenous Cnp1 nucleosomes do not fully occupy the centromeres (see the legend to Fig. 2 for details).

Cnp1-GFP (S65T) cells exhibit mild temperature sensitivity in growth, and it was indicated that the Cnp1-GFP (S65T) fluorescent signal may be diminished (25). To test whether the GFP signal diminishing is a temperature-dependent phenomenon, we compared the GFP signal intensity in live Cnp1-GFP (S65T) cells at two temperatures: 25 °C (at which Cnp1-GFP (S65T) cells grow at the wild type rate) and 32 °C (at which the published measurement was conducted, indicating diminishment in the GFP signal in Cnp1-GFP (S65T) cells). Cnp1-mEGFP cells, which do not exhibit temperature sensitivity in growth, were also tested as a control. Indeed, in Cnp1-GFP (S65T) cells, the GFP signal was significantly reduced at 32 °C compared with 25 °C. In Cnp1-mEGFP cells, the reduction in the GFP signal intensity was mild (Fig. 2C). To minimize the impact of temperature on the measurement, all of the GFP signal measurement for the purpose of quantifying Cnp1-GFP (S65T) copy number was conducted at 25 °C, and the Ndc80-GFP (S65T) was used as the reference (39).

Thus, although our biochemical analysis of a cell population indicates a nearly complete occupancy of centromere cores by Cnp1, quantitative fluorescence microscopy studies suggest a partial occupancy by Cnp1. To resolve the apparent discrepancy, we postulate that the positions occupied by Cnp1 nucleosomes vary among individual cells within a genetically homogeneous cell population.

Centromeres Tolerate Substantial Reduction in the Length of Central Core—If the Cnp1 nucleosomes are only a portion of the total nucleosomes within the centromeric core and their positions are variable, it is possible that the centromeric core DNA may not require its full length to support the assembly of a functional kinetochore. Previous studies have indicated that no specific centromeric DNA sequence is essential (12). We sought to test whether centromeric DNA can be reduced in length without compromising the essential biological functions of the centromere. We engineered truncations of the endogenous cen1 (excision of cnt1 only or together with part of imr1R, causing reduction in the length of the centromeric core by 48 or 58%, respectively). Cells with truncated cen1 grew at the wild type rate under normal conditions (Fig. 3). With a similar strategy, we also engineered the endogenous cnt2 by truncation up to 50% of the total cnt2, without causing any noticeable change in the rate of cell growth (3.29-kb cc2 region, (1,624,217–1,627,510 of chromosome II) was deleted; data not shown). Chromatin immunoprecipitation using anti-Cnp1 confirmed that both shortened cnt1 and cnt2 bind to Cnp1 (Fig. 3D), indi-
indicating that they function as a centromere. Furthermore, we challenged cnt1 and cnt2 truncation with a microtubule-destabilizing chemical (thiabendazole) that is toxic for mitosis. Both truncations grew at the wild type rate (Fig. 3E). Together, these results suggest that an “oversized” centromeric core DNA is a general feature of S. pombe centromeres.

FIGURE 3. Truncated cnt1 is sufficient to support normal cell growth. A, schematic representation of centromere I and its engineered version designed for excision of cnt1 or cnt1-imr1R region. The red box and red arrow indicate the RS site and region excised by R recombinase, respectively. The green lines indicate the positions of the SphI site. Black horizontal lines with a number represent the length of fragments hybridized by DNA probe (imr1 region, marked by blue box). B, DNAs prepared from wild type, cnt1-excised, or cnt1-imr1R-excised clones were digested by SphI and hybridized with the probe in the imr1 region. C, serial dilutions (10 times) of the indicated cells were spotted onto a YEA plate and were incubated at 30 or 26 °C for 2 or 3 days, respectively. D, ChIP using anti-Cnp1 antiserum (gift from R. Allshire) on strains carrying a truncated cnt2 or cnt1. DNAs recovered from WCE or immunoprecipitated products (ChIP) were tested. E, cells with a shortened centromere exhibit a normal level of resistance to thiabendazole (TBZ). Serial dilutions of wild type cells or cells with a truncated centromere (as labeled) were spotted onto YEA plates containing thiabendazole at the labeled levels and were incubated at 26 °C for 3 days.
Evidence against the Model of Silencing within the Centromeric Core Due to Heterochromatin Spreading—Earlier studies have demonstrated that expression of a reporter gene in the centromeric core is variegated in a genetically homogenous population (32). Genetic evidence shows that mutations in \textit{cnp1}^{+} or genes affecting Cnp1 deposition in centromere reduce the level of silencing of the reporter gene (32, 40 – 43), indicating a possible link between Cnp1 incorporation and gene silencing. This is in good agreement with the notion that variable Cnp1 positioning, as indicated by the above evidence (Fig. 1), underlies PEV in the centromere. An alternative possible mechanism explaining the PEV in the centromeric core is that the heterochromatin status of the pericentromeric regions may occasionally spread into the core, causing gene silencing. In other words, the boundary between the centromeric core and the pericentromeric regions imposed by sets of tRNA genes (44) may be breached stochastically.

To distinguish between these two possibilities and better characterize the mechanism of gene silencing in the centromeric core, we engineered \textit{cnt2} in its native chromatin context by inserting two reporter genes, \textit{ade6}^{+} and \textit{ura4}^{+}, simultaneously. For the \textit{ade6}^{+} reporter, the transcriptional states can be readily visualized by the color of the colonies grown on medium containing limited adenine supply; \textit{ade6}^{+}-OFF leads to the formation of red colonies, whereas \textit{ade6}^{+}-ON cells form white colonies. For the \textit{ura4}^{+} reporter, its expression states can be selected by specific medium conditions; only \textit{ura4}^{+}-OFF cells will grow on medium containing 5FOA, and only \textit{ura4}^{+}-ON cells will grow on media lacking uracil (32). The two reporters were inserted in tandem at one end of \textit{cnt2}, with only about 200 bp of space between (the “tandem” construct in Fig. 4, top). We reason that if the silencing state spreads from the pericentromeric heterochromatin toward the middle of \textit{cnt2}, the directionality of the spreading would be manifested by a unilateral, co-silencing constraint of the two reporters. Specifically, in the “tandem” construct, when the internal \textit{ura4}^{+} gene is silenced, the external \textit{ade6}^{+} should be invariably silenced. However, under either condition of \textit{ura4} ON (uracil dropout medium) or OFF (5FOA medium), colonies were found exhibiting white or red color (Fig. 4, bottom), indicating that \textit{ade6}^{+} could be silenced or expressed when \textit{ura4}^{+} was silenced, arguing against the directional spreading of the heterochromatin inward to the centromeric core.

\textbf{Cnp1 Occupancy Directly Correlates with Centromeric Core Silencing}—To directly test whether gene silencing is linked to Cnp1 occupancy, we measured the levels of Cnp1 occupancy in cells with two reporters in “tandem” positions, either at the ON or OFF conditions for both genes. We first compared Cnp1 occupancy on multiple loci at the endogenous \textit{cnt2} sequence, \textit{ura4}^{+} and \textit{ade6}^{+}, using quantitative PCR (Fig. 5). Although similar levels of Cnp1 occupancy were detected on loci within the endogenous \textit{cnt2} sequence, elevated levels of Cnp1 were found both on \textit{ura4}^{+} and \textit{ade6}^{+} reporters in cells with the reporters silenced relative to that in cells with no selection. To compare Cnp1 occupancy systematically at all possible loci, we performed Cnp1 mononucleosome immunoprecipitation combined with high throughput sequencing in cells with actively transcribed or silenced reporters in centromere 2. The results demonstrate that on the endogenous \textit{cnt} DNA elements, normalized Cnp1 occupancy was nearly identical between the two cell populations. In contrast, higher levels of Cnp1 nucleosomes were found on all of the nucleosome positions at \textit{ura4}^{+} and \textit{ade6}^{+} under the gene-silenced condition relative to the active condition (Fig. 6, A and B). No significant difference in overall nucleosome occupancy at the \textit{ade6}^{+} cassette was detected between the two conditions, as determined by total nucleosome high throughput sequencing (Fig. 6C). Difference in Cnp1 occupancy on the reporter genes at different transcription status (ON or OFF) was confirmed by independent chromatin immunoprecipitation and qPCR experiments (data not shown). Together, these results demonstrate that elevation in Cnp1 nucleosome occupancy correlates with silencing of the reporter genes in the centromere.

\textbf{Evidence for Epigenetic Inheritance of Cnp1 Positioning and Quantification of the Rates of Its Switching by the Pedigree Analysis Assay}—Because Cnp1 occupancy directly correlates with gene silencing, quantification of variegation in gene expression would serve as a useful tool for the assessment of dynamics in Cnp1 nucleosome positioning. We reasoned that for any single cell carrying a centromeric \textit{ade6}^{+} reporter, the cell has a specific Cnp1 localization pattern, corresponding to a distinct \textit{ade6}^{+} expression state. Whether its progeny maintain the same expression state or switch to an alternative state should indicate whether the Cnp1 localization pattern on \textit{ade6}^{+} is inherited or switched between the cell generations.

We designed a pedigree analysis assay to track the \textit{ade6}^{+} expression through cell generations. For each progenitor cell, multiple (typically three) generations were tracked under a microscope. By micromanipulation, progenies after each round of cell division were placed at designated sites on the solid medium surface so that their pedigree information was preserved. The progenies of the final cell generation were allowed to grow, forming colonies. We found that within each pedigree, the progeny colonies usually displayed a highly similar coloration, suggesting that the levels of \textit{ade6}^{+} expression and, therefore, the Cnp1 nucleosome positioning are inherited (Fig. 7A;
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A.

| qPCR Amplicons | endogenous (1,2,3) | URA (1) | ADE (1,2,3) | Neg. Ctrl. |
|----------------|--------------------|---------|-------------|------------|
| uRA4           | ade6               |         |             |            |
| ade6           | uRA4               |         |             |            |

B.

Figure 5. Enrichment of Cnp1 measured by qPCR correlates with reporter gene silencing in cnt2. A, schematic illustration of positions of the PCR primer pairs (amplicons) used for quantitative PCR analysis. Multiple pairs of primers were used: endogenous cnt2 (green), three pairs; ura4 reporter (brown), one pair; ade6 reporter (red), three pairs. One pair corresponding to a chromosome arm locus (blue) was used as the negative control. B, quantitative comparison of Cnp1 occupancy at various loci between the conditions of reporter genes silenced or not under selection. Loci are as illustrated above. For each locus, folds of Cnp1 enrichment relative to the negative control (y axis, left) were determined at both gene-silenced (open columns) and non-selective (dashed columns) conditions.

We also measured the rates of switch in cells with cnt2 overexpression, or mutations in genes that were known or speculated to affect Cnp1 deposition. We measured the rates for both red-to-white switch and white-to-red switch (Cnp1 translocation away and onto ade6+). We first measured the rates of switch in cells carrying the same ade6+ construct but with a truncation of 50% of the original cnt2 (Fig. 6B). We measured approximately a 3- and 2-fold reduction in the rates of red-to-white switch and white-to-red switch, respectively, in comparison with wild type, demonstrating that the rates of switch, indeed, are a property of the centromeric chromatin and correlate with the length of the central core.

We also measured the rates of switch in cells with cnt2 overexpression, which previously has been shown to cause DNA sequence has little influence on Cnp1 nucleosome deposition, an epigenetic mechanism must guide the new Cnp1 deposition after DNA replication, using the existing Cnp1 as the spatial cue, so that the cell maintains the “memory” of Cnp1 nucleosome positioning at the single nucleosome level. Such epigenetic memory, however, is prone to change through generations of cell division at a marked level in wild type cells.

Characterization of Mutations That Affect the Rates of Switching in Cnp1 Positioning—We sought to explore what may influence or modulate the plasticity of gene silencing in the centromeric core. Based on the evidence that silencing in the central core is unlikely to be due to spreading from the flanking heterochromatin regions (Fig. 4), the underlying mechanism(s) is expected to be distinct from gene silencing at the heterochromatin regions via siRNA and histone H3 Lys-9 methylation (20, 45). We tested candidate genetic alterations for their effects on altering the rates of switch, including a centromeric core truncation, cnt2+ overexpression, or mutations in genes that were known or speculated to affect Cnp1 deposition. We measured the rates for both red-to-white switch and white-to-red switch (Cnp1 translocation away and onto ade6+).
increased incorporation of Cnp1 in centromeres (10). We found that Cnp1 overexpression causes enhanced silencing; nearly all cells with cnp1/H11001 overexpression formed red colonies with a different shade of color. White colonies were sparse (4–5/10^3). Consistently, the rate of red-to-white switch reduced to 2.6%, compared with 6.8% in wild type cells (Fig. 7B). We were not able to measure white-to-red switching due to the rarity of such events. This result supports the notion that Cnp1 occupancy leads to gene silencing.

We further explored the potential impact of deletions of genes encoding chromatin remodeling factors or histone chaperones on Cnp1 position inheritance. We observed a significant increase in the rates of switching (for both red to white and white to red) in cells that have a deletion of pcf3, which encodes for a subunit of the CAF-1, a protein complex that functions in replication-coupled nucleosome assembly (46). Conversely, deletion of vps75, which encodes for a histone chaperone protein (47), causes significant reduction in the rates of switching (Fig. 7B). Studies in budding yeast have shown that Vps75 forms a complex with a histone acetyltransferase, Rtt109, and enhances the activity of Rtt109 in acetylating lysine (Lys-56) of histone H3 (48, 49), or Vps75 may function outside the context of the acetyltransferase enzyme complex and modulate the expression of a large host of genes (50). To test whether histone
H3 Lys-56 acetylation is involved in modulating Cnp1 positioning, we measured the switch frequency in rtt109Δ/H9004 cells. rtt109Δ/H9004 cells exhibited the wild type level of switching (Fig. 7B), suggesting that Vps75 modulates the rates of switch via a mechanism other than histone H3 Lys-56 acetylation. These results together suggest that Cnp1 nucleosome inheritance and repositioning are modulated by specific genetic pathways. Elucidation of the underlying biochemical mechanisms requires further investigation.

**DISCUSSION**

In this study, we have made several novel findings in chromatin organization of the small regional centromere in the fission yeast. First, the Cnp1/CENP-A nucleosomes, accounting for a portion of the total nucleosomes at the central core of the centromere, can localize to nearly all of the sites throughout the central core. Second, the endogenous central core region is larger than what is needed for the essential role in supporting mitotic cell division. Third, Cnp1 occupancy directly correlates to the silencing of underlying reporter genes, providing the explanation for the previously observed PEV at the centromeric core. Fourth, for individual cells, positioning of Cnp1, reflected by reporter gene silencing, can be inherited through cell divisions, although repositioning also occurs at marked rates. Finally, inheritance of Cnp1 positioning as well as its repositioning involve specific proteins such as (and probably not limited to) histone chaperones CAF-1 and VPS75. Together, these conclusions support a model of centromere organization, highlighting the plasticity and epigenetic inheritance of CENP-A nucleosome positioning (Fig. 8). Furthermore, they also provide the necessary framework based on which further investigations of fission yeast centromere identity establishment and propagation will be performed.

We present direct evidence that under physiological conditions, Cnp1 occupancy correlates with gene silencing. This is in good agreement with the earlier genetic evidence that overall reduction in Cnp1 occupancy due to mutations in specific kinetochore proteins correlates with enhanced expression of reporter genes in the centromere (10, 41, 51). Conversely, overexpression of cnp1+, leading to elevated incorporation of Cnp1 in the centromere, causes enhanced gene silencing (37). In our assay, we also tested the effect of Cnp1 overexpression and consistently found that nearly all cells carrying the centromeric ade6 marker form red colonies under conditions of Cnp1

![Figure 7](image-url)

**FIGURE 7. Quantification of the rates of switching of the cnt2::ade6 reporter by pedigree analysis.** A, schematic diagram of the pedigree analysis procedure and examples of cell pedigrees. A progenitor cell (generation I) and its progenies of three generations were micromanipulated to designated positions on solid growth medium to preserve the pedigree information (top diagram). The identity of the cells of earlier generation (dashed line ovals) was tracked, but these cells disappeared after giving birth to the cells of the next generation. Cells of the last generation (solid line ovals) grew to form colonies. Relationship (i.e. sisters, close cousins, distant cousins) was preserved by the relative positions of the colonies on the plate. Shown below are examples (1–3 in white) of pedigrees. Each row of colonies is one pedigree. B, quantification of the rates of red-to-white and white-to-red switching in mutant strains (total cell divisions scored: n >500). The S.D. (error bars) is calculated by clustering cell families randomly into three or four subgroups, each containing about 200 cell division events. C, more examples of wild type cell families. Each row of colonies represents a three-generation cell family that is tracked by the pedigree analysis. The circle highlights a cell (or its descendents) switched from red to white (top row) or white to red (second row). The rare cases with equal numbers of red and white colonies (third row) are consistently scored as red-to-white switch. The bottom two rows exemplify red or white (light pink) pedigrees with no switching.
Plastic CENP-A Positioning and Its Epigenetic Inheritance

![Diagram: Model for flexible Cnp1 nucleosome positioning and its inheritance in fission yeast centromere.](Image)

Our characterization of centromeric PEV through several cell generations provides direct evidence that Cnp1 occupancy is inheritable near the single nucleosome level; there are a maximum of six nucleosomes in the ade6+ cassette, and our current study does not clearly distinguish different levels of Cnp1 occupancy on ade6+. Meanwhile, Cnp1 nucleosome positioning also undergoes switching through cell generations at marked rates. The fact that both expression states, ON or OFF, of ade6+ are inheritable suggests that gene transcription per se does not determine the inheritance or the switch of the chromatin organization. This, however, does not exclude the possibility that gene transcription may quantitatively influence the inheritance of the chromatin structure. Thus, caution needs to be taken to extrapolate the level of precision in chromatin duplication based on the characterization of specific reporter genes (e.g. the pedigree analysis of centromeric PEV based on ade6+ expression). Nonetheless, the pedigree analysis should be useful in the future study to assess the possible impact by individual proteins (such as Vps75 and CAF-1) among the rich repertoire of chromatin-related protein factors.

In metazoans, centromeric chromatin is postulated to fold into elaborate patterns (9, 29, 30), so that the linearly dispersed CENP-A nucleosome patches are clustered at one side of the three-dimensional centromere structure. Such a configuration would be important to reduce the chance of merotelic attachment between the kinetochore and the spindle microtubules (i.e. one kinetochore is erroneously attached to microtubules originated from both ends of the spindle).

It is conceivable that in the relatively simple centromere in fission yeast, the same requirement is also imposed to orient Cnp1 nucleosomes toward the same end of the spindle. We envision two specific models, both fitting the stochastic Cnp1 positioning on an oversized centromeric core. One postulates that Cnp1 nucleosomes are localized on the centromere, with highly flexible distribution patterns so long as they fulfill the requirement of being clustered on one side of the centromeric chromatin high order structure; the other model postulates that Cnp1 nucleosomes may be locked into a specific pattern relative to each other (e.g. alternating with H3 nucleosomes), forming the base for kinetochore assembly. The kinetochore base as a whole entity may have limited flexibility in positioning within the centromere. Due to the considerable variation in Cnp1 positioning in wild type cells, our current results of Cnp1 positioning represent the cell population average and lack sufficient resolution to discern specific organization patterns of Cnp1 to distinguish between these two models.

Recently, Lando et al. (25) used ChIP-seq analysis to map the Cnp1-containing nucleosome and quantified the Cnp1 protein copy number within the centromere cluster of single cells. Their results overall are consistent with the notion that not all Cnp1 nucleosomes in individual cells occupy the same positions. In terms of the relative abundance of the nucleosome peaks (in cnt2), the work of Lando et al. (25) differs quantitatively from our results (Fig. 1). In particular, Lando et al. (25) showed two strongly preferred sites (accommodating three nucleosomes in total) within Cnt2, in contrast to our results, in which the occupancy levels on all Cnp1 sites are comparable. We are unsure what causes the discrepancy, but we speculate that this may be due to difference in experimental conditions. Noticeably, in the work of Lando et al. (25), cells were cultured at 32 °C, a condition under which the PEV phenomenon ceases to exist, whereas in all of our experiments, cells were cultured at 25 °C, under which PEV is prominent.

overexpression. Correlation between CENP-A occupancy and gene silencing is also reported in Candida albicans neocentromeres, the newly formed functional centromeres at ectopic sites upon loss of the endogenous centromere (52), and in C. elegans, in which CENP-A is distributed broadly throughout the whole chromatin but inversely correlating with transcription of the underlying chromatin regions in germ line (2). Also, reduction or increase in the Cnp1/histone H3 ratio led to reduced or enhanced gene silencing, respectively, in centromere (37). Thus, it appears to be a common property in various species that CENP-A localization causes silencing in gene expression. On the other hand, it remains possible that gene expression may in turn influence Cnp1 incorporation. Overall, our finding in normal fission yeast cells provides the underlying mechanism for the position effect variegation phenomenon in the centromere, revealing the dynamic aspect of centromeric chromatin structure. Considering that transcription does occur in the centromere, producing non-coding RNA (53), how transcription and chromatin organization may influence each other (perhaps by forming a positive feedback loop for stable CenpA nucleosome inheritance) is a topic of fundamental importance for centromere biology that requires future in depth investigation.

The epigenetic nature of centromeric chromatin propagation was initially recognized based on the fact that in most organisms (except for the budding yeast S. cerevisiae and several other closely related yeasts) the centromere identity is not determined by specific DNA sequence (54, 55). Instead, CENP-A nucleosomes are postulated to be the epigenetic marker of centromeric chromatin. On the other hand, studies in C. elegans embryos showed that in cell divisions, parental CeCENP-A molecules undergo complete turnover after replication. A spatial cue for new CeCENP-A incorporation, instead, is provided by the transcription activity in the germ line (2). Thus, the CENP-A nucleosome inheritance process is unlikely to be conserved in this organism with holocentromeres.

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Interestingly, we observed that when Cnp1 occupancy was reduced on the reporter genes correlating with gene expression, there was no corresponding increase in Cnp1 occupancy on the remaining endogenous Cnt2 region (Fig. 6B). Thus, despite the fact that in wild type cells, a constant Cnp1 molecule copy number was measured within centromeres, the level of Cnp1 incorporation is flexible and can be reduced (due to transcription of the underlying genes) or increased (due to overexpression of cnp1).

Compared with wild type cells, fission yeast cells with a shortened centromere do not exhibit readily noticeable difference in cell growth rates, either under the optical growth conditions or when challenged with a chemical (thiabendazole) that destabilizes microtubules and is toxic for mitosis (Fig. 3E). This suggests that flexibility in Cnp1 positioning may not have an essential functional role in vegetative cell growth. This is consistent with our observation that relocation of Cnp1 within the centromere only occurs at a low (despite being significant) frequency. Our current test, however, does not detect a subtle reduction in fidelity of chromosome segregation. Thus, a subtle, quantitative effect on each cell cycle under specific physiological conditions (stresses, for example) cannot be ruled out, it is possible that the flexibility in Cnp1 positioning is closely related to the lack of centromeric DNA sequence conservation among different organisms and lack of high sequence identity among different centromeres in the same organism as well as the capability of forming a neocentromere upon loss of the endogenous centromere. Each chromosome needs to form one and only one centromere. It is conceivable that rigid sequence specificity for Cnp1 positioning, although perhaps being effective in ensuring one centromere per chromosome, carries high risks of centromere loss with DNA mutations because such stringency would be incompatible with neocentromere formation at an alternative locus. Indeed, neocentromere formation in fission yeast is known to occur in a broad region (20 kb) near the telomere that is low in gene expression (56). These characteristics of neocentromere formation are consistent with the flexibility of Cnp1 positioning in endogenous centromeres and may indicate that the same mechanism is employed for the formation of both endogenous and neo-centromeres. Because neocentromeres form broadly in eukaryotes, including mice and humans, it is possible that flexibility of CENP-A positioning is also a common phenomenon. Future studies are required to elucidate the molecular mechanisms of the inheritance and the switch of CENP-A position and how the process of CENP-A deposition is integrated with other mitotic processes to achieve the assembly of only one centromere per chromosome to maximize the fidelity of chromosome segregation.

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