Ectopic Centromere Nucleation by CENP-A in Fission Yeast

Marlyn Gonzalez, Haijin He, Qianhua Dong, Siyu Sun, and Fei Li[1]
Department of Biology, New York University, New York, New York 10003

ABSTRACT The centromere is a specific chromosomal locus that organizes the assembly of the kinetochore. It plays a fundamental role in accurate chromosome segregation. In most eukaryotic organisms, each chromosome contains a single centromere the position and function of which are epigenetically specified. Occasionally, centromeres form at ectopic loci, which can be detrimental to the cell. However, the mechanisms that protect the cell against ectopic centromeres (neocentromeres) remain poorly understood. Centromere protein-A (CENP-A), a centromere-specific histone 3 (H3) variant, is found in all centromeres and is indispensable for centromere function. Here we report that the overexpression of CENP-A^{Cnp1} in fission yeast results in the assembly of CENP-A^{Cnp1} at noncentromeric chromatin during mitosis and meiosis. The noncentromeric CENP-A preferentially assembles near heterochromatin and is capable of recruiting kinetochore components. Consistent with this, cells overexpressing CENP-A^{Cnp1} exhibit severe chromosome missegregation and spindle microtubule disorganization. In addition, pulse induction of CENP-A^{Cnp1} overexpression reveals that ectopic CENP-A chromatin can persist for multiple generations. Intriguingly, ectopic assembly of CENP-A^{cnp1} is suppressed by overexpression of histone H3 or H4. Finally, we demonstrate that deletion of the N-terminal domain of CENP-A^{cnp1} results in an increase in the number of ectopic CENP-A sites and provide evidence that the N-terminal domain of CENP-A prevents CENP-A assembly at ectopic loci via the ubiquitin-dependent proteolysis. These studies expand our current understanding of how noncentromeric chromatin is protected from mistakenly assembling CENP-A.

THE centromere is a specific chromosomal locus that organizes the assembly of the kinetochore. It is vital for the proper segregation of chromosomes during mitosis and meiosis (Allshire and Karpen 2008; Henikoff and Furuyama 2010). Most eukaryotic chromosomes contain a single centromere that is faithfully inherited at the same position within the chromosome through generations. In "point" centromeres of *Saccharomyces cerevisiae*, a specific 125-bp DNA sequence is both necessary and sufficient to specify centromere position (Clarke and Carbon 1980; Cottarel *et al.* 1989). However, most other eukaryotes contain regional centromeres, which are more complex and usually consist of large blocks of repetitive DNA sequences (Pluta *et al.* 1995; Henikoff *et al.* 2001). Epigenetic mechanisms appear to play a dominant role in the formation and inheritance of regional centromeres (Allshire and Karpen 2008; Henikoff and Furuyama 2010).

Copyright © 2014 by the Genetics Society of America
doi: 10.1534/genetics.114.171173
Manuscript received June 5, 2014; accepted for publication September 27, 2014; published Early Online October 7, 2014.
Supporting information is available online at http://www.genetics.org/lookup/suppl/doi:10.1534/genetics.114.171173/-/DC1.
[1]Corresponding author: Department of Biology, New York University, New York, NY 10003. E-mail: fl43@nyu.edu

Centromere protein-A (CENP-A), a centromere-specific histone 3 (H3) variant, has been proposed to act as the epigenetic mark for centromere positioning (Palmer *et al.* 1991; Henikoff and Furuyama 2010; Burrack and Berman 2012; Muller and Almouzni 2014). CENP-A partially replaces canonical histone H3 at the centromere and provides the structural and functional foundation for the assembly of the kinetochore (Black and Cleveland 2011; Maddox *et al.* 2012). The assembly of CENP-A at regional centromeres can occur independently of the underlying DNA sequence. This is evidenced by the observation that *de novo* centromeres (neocentromeres) can form at noncentromeric regions in a variety of organisms (Murphy and Karpen 1995; du Sart *et al.* 1997; Maggert and Karpen 2001; Hiatt *et al.* 2002; Warburton 2004; Marshall *et al.* 2008; Ketel *et al.* 2009; Topp *et al.* 2009; Zhang *et al.* 2010; Fu *et al.* 2013; Shang *et al.* 2013; Scott and Sullivan 2014). In addition, CENP-A is overexpressed in many cancer cells, and this results in the incorporation of CENP-A at noncentromeric loci (Tomonaga *et al.* 2003; Li *et al.* 2007; Amato *et al.* 2009; Scott and Sullivan 2014). In *Drosophila*, overexpression of the CENP-A homolog, CID, can lead to ectopic formation of heritable functional kinetochores and significant chromosome missegregation (Heun

et al. 2006; Olszak et al. 2011). In budding yeast and *Drosophila*, ubiquitin-mediated proteolysis of CENP-A has been shown to contribute to the removal of mislocalized CENP-A at noncentromeric regions (Collins et al. 2004; Moreno-Moreno et al. 2006; Hewawasam et al. 2010; Ranjitkar et al. 2010). However, the mechanisms that protect cells from erroneously assembling CENP-A at noncentromeric chromatin remain poorly understood.

The fission yeast *Schizosaccharomyces pombe* contains regional centromeres like those found in humans and other eukaryotes; thus it represents an excellent model for studying general principles of eukaryotic centromere regulation (Westermann and Schleiffer 2013). In the fission yeast centromere, the CENP-A homolog Cnp1 (CENP-A^{cnp1}) associates with 10–12 kb of the central domain region that is flanked by peri-centromeric heterochromatin (Allshire and Karpen 2008). This centromere architecture is highly conserved among organisms with regional centromeres (Carroll and Straight 2006; Westermann and Schleiffer 2013). In additon to peri-centromeric heterochromatin, telomeres and mating-type loci are also heterochromatic in nature. These constitutive heterochromatin regions are regulated by the methylation of histone H3 at lysine 9 (H3K9me), which serves as a binding site for the chromodomain protein Swi6, the homolog of human HP1 (Nakayama et al. 2001; He et al. 2014).

In fission yeast, deletion of native centromeric DNA results in the assembly of a functional ectopic centromere at noncentromeric chromatin (Ishii et al. 2008). Recently, it has also been shown that overexpression of CENP-A^{cnp1} causes the promiscuous incorporation of CENP-A^{cnp1} near heterochromatic regions and the deleterious effect on cell growth (Choi et al. 2012; Castillo et al. 2013). However, it remains unclear whether ectopic CENP-A^{cnp1} chromatin triggered by the overexpression of CENP-A^{cnp1} is able to direct the assembly of functional kinetochores and how ectopic CENP-A^{cnp1} chromatin is regulated. Here we show that, in agreement with previous findings (Choi et al. 2012; Castillo et al. 2013), overexpression of CENP-A^{Cnp1} in mitotic cells results in growth defects and the assembly of CENP-A^{Cnp1} at noncentromeric chromatin, preferentially at the nuclear periphery and within heterochromatin boundary regions. We further show that the ectopic CENP-A^{cnp1} chromatin is stable for multiple generations and that a subset of ectopically assembled CENP-A^{cnp1} islands are able to recruit kinetochore components. Assembly of functional ectopic kinetochores at these sites is further supported by our observation that cells overexpressing CENP-A^{Cnp1} exhibit severe chromosome segregation defects and spindle disorganization. Intriguingly, we show that the N-terminal domain of CENP-A^{Cnp1} plays a role in excluding CENP-A^{Cnp1} from noncentromeric chromatin by mediating ubiquitin-dependent proteolysis of CENP-A^{cnp1}. Furthermore, we report the novel findings that formation of ectopic CENP-A^{Cnp1} chromatin can be suppressed by overexpression of either histone H3 or H4. Finally, we provide the first evidence that, in meiotic cells, as in mitotic cells, the overexpression of CENP-A results in the assembly of CENP-A at noncentromeric chromatin and concomitant chromosome segregation defects. These studies support CENP-A^{Cnp1} as the epigenetic mark for centromere identity and lay the foundation for future studies on ectopic centromere assembly in eukaryotes.

Materials and Methods

Strains, media, and DNA constructs

Fission yeast strains used in this study are listed in the Supporting Information, Table S1. Standard media and genetic analysis for fission yeast were used (Moreno et al. 1991). Full-length histones H3 and H4 were constructed in the pREP2 vector containing the *nmt1* promoter. CENP-A^{cnp1}, $Cnp1^{CATD\Delta}$, and $Cnp1^{N\Delta}$ were cloned into the pREP1 or pREP2 vector containing in-frame GFP or mCherry.

In situ chromatin-binding assay

In situ chromatin-binding assays were performed as described previously (Kearsey et al. 2000). Briefly, exponentially growing cells were collected and incubated at 32° in ZM buffer (50 mM sodium citrate pH 5.6, 1.2 M sorbitol, 0.5 mM MgAc, 10 mM dithiothreitol) for 30 min. Cells were washed with EB buffer (20 mM PIPES–potassium hydroxide, pH 6.8, 0.4 M sorbitol, 2 mM MgAc, 150 mM KAc) containing 1% Triton X-100 at 20° for 7 min. Following fixation with 3.7% formaldehyde and 10% methanol, cells were examined by epifluorescence microscopy.

Pulse overexpression of CENP-A

Cells carrying pREP2-CENP-A^{cnp1}-GFP or pREP2-CENP-A^{cnp1}-mCherry were incubated for 24 hr in the minimum media without thiamine to induce overexpression of CENP-A^{cnp1}. Thiamine was subsequently added to a final concentration of 100 μM to repress overexpression of CENP-A^{cnp1}. The distribution pattern of CENP-A^{cnp1}-GFP and CENP-A^{cnp1}-mCherry was monitored for multiple generations after the addition of thiamine by fluorescence microscopy.

Microscopy

Cells were imaged using the Delta Vision System (Applied Precision, Issaquah, WA). Images were taken as z-stacks of 0.2-μm increments with an oil immersion objective (×100) and deconvolved using SoftWoRX2.50 software (Applied Precision). Standard DAPI staining and analysis methods for fission yeast nuclei were used.

Western blot analysis

Cell extracts from 2×10^8 exponentially growing cells were prepared using standard protocols and a bead beater. Extract proteins were separated on 12% SDS-polyacrylamide gels and blotted onto PVDF membranes. Standard Western blot protocols were used in subsequent steps. Blots were probed with anti-GFP (Roche, 11 814 460 001) or α-tubulin (Abcam, ab6160) antibodies.

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) assays were performed as described (Li *et al.* 2008). Briefly, cells were grown to log phase at 22° and treated with 1% formaldehyde to generate DNA-protein cross-linking. Immunoprecipitation was performed using an anti-GFP antibody. Precipitated DNA was suspended in 30 μl Tris-EDTA buffer. Two microliters of ChIP or whole-cell extracts (WCEs) samples were analyzed by competitive PCR using oligonucleotides specific to a telomere boundary region and to the control gene *act1*$^+$. Twenty-seven to 30 amplification cycles were used for each PCR reaction. PCR products were separated in 1.7% agarose gels and post-stained with ethidium bromide. Primers used are listed in Table S2. All experiments were conducted in triplicate. Quantitations were performed using ImageJ 1.46r software.

Ubiquitin affinity pull-down assays

Ubiquitinated CENP-A^{cnp1}-GFP was pulled down using Tandem Ubiquitin Binding Entities (TUBEs) (Lifesensors) according to the manufacturer's instructions. Briefly, 50 ml of exponentially growing cells were treated with MG132 (20 μM, Selleck Biochemicals) for 3 hr prior to collection. Extracts from these cells were then incubated in the presence of TUBE agarose or control beads at 4° for 1 hr. Proteins eluted from washed beads were analyzed by Western blotting using an anti-GFP antibody (Roche, 11 814 460 001).

Results

Overexpression of CENP-A^{cnp1} results in assembly of CENP-A^{cnp1} at ectopic loci

To investigate whether overexpression of CENP-A^{cnp1} results in assembly of CENP-A chromatin at noncentromeric regions, we constructed CENP-A^{cnp1}-GFP under the strong thiamine-repressible *nmt1* promoter. In minimal media containing 100 μM thiamine, the uninduced *nmt1* promoter displays leaky expression of CENP-A^{cnp1}-GFP near its endogenous level (Figure 1, A and B). Intermediate and full induction of the promoter is achieved by supplementing the medium with 0.05 μM thiamine or no thiamine at all for 24 hr, at which time the protein level increases by ~60- and 200-fold, respectively (Figure 1B and Figure S1). In wild-type interphase fission yeast cells, centromeres are clustered together at the nuclear envelope adjacent to the spindle pole body (SPB). This results in the formation of a single fluorescent focus at the nuclear envelope in cells expressing CENP-A^{cnp1}-GFP at the endogenous level (Gonzalez *et al.* 2013). To further determine whether the GFP-tagged CENP-A^{cnp1} is functional, we replaced endogenous CENP-A^{cnp1} with CENP-A^{cnp1}-GFP. Cells carrying CENP-A^{cnp1}-GFP at its endogenous site are viable and grow only slightly slower than wild-type cells expressing untagged CENP-A^{cnp-1} (Figure S2). This result coincides with the previous studies (Takayama *et al.* 2008; Lando *et al.* 2012), indicating that CENP-A^{cnp1}-GFP is largely functional. Consistent with this, these cells display a single fluorescent focus at the nuclear envelope (data not shown).

Like endogenously expressed CENP-A^{cnp1}-GFP, CENP-A^{cnp1}-GFP expressed from uninduced *nmt1* promoter exclusively targets to centromeres, displaying a single fluorescent focus (Figure 1A). In contrast, >96% of the cells expressing CENP-A^{cnp1}-GFP from intermediately induced or fully induced *nmt1* promoter display multiple fluorescent foci, indicating that CENP-A^{cnp1}-GFP mislocalizes to noncentromeric chromatin in these cells (Figure 1A). In addition, cells with full induction of CENP-A^{cnp1}-GFP generally contain more fluorescent foci (more than threefold) than cells with intermediate induction, and the foci in fully induced cells also appear elongated as if spreading farther along the chromosome (Figure 1A and Figure S3).

To determine whether ectopically localized CENP-A^{cnp1}-GFP is chromatin-bound, we performed *in situ* chromatin-binding assays (Kearsey *et al.* 2000). After extensive washing with the non-ionic detergent Triton X-100, excessive fluorescent foci could be still observed in cells overexpressing CENP-A^{cnp1}-GFP (Figure S4). As a control, we used the *dos1*Δ mutant expressing GFP-Swi6 at the endogenous level. In wild-type cells, the heterochromatin marker GFP-Swi6 forms two to six distinct fluorescent foci corresponding to heterochromatin regions. In the *dos1*Δ mutant, GFP-Swi6 fails to associate with chromatin (Li *et al.* 2005) and is therefore effectively washed away from the nucleus by Triton X-100 (Figure S4). These observations demonstrate that excessive CENP-A^{cnp1}-GFP is able to bind noncentromeric chromatin, consistent with previous findings (Choi *et al.* 2012; Castillo *et al.* 2013).

Ectopic CENP-A^{cnp1}-GFP preferentially assembles near the nuclear periphery and heterochromatin in mitotic cells

We next asked where in the genome CENP-A^{cnp1}-GFP assembles when overexpressed. Fluorescent microscopy analysis indicated that ectopic CENP-A^{cnp1}-GFP preferentially associates with the nuclear periphery. This is most clear when CENP-A^{cnp1}-GFP expression is induced at the intermediate level (Figure 1A). To further confirm this, we overexpressed CENP-A^{cnp1}-GFP in cells carrying the mCherry-tagged nuclear envelope protein, Ish1-mCherry (Asakawa *et al.* 2010). We found that in >90% of cells induced at intermediate level, GFP signal is enriched at the nuclear periphery (Figure 1C). The nuclear periphery is generally regarded as a transcriptionally repressive environment. In *S. pombe*, all three heterochromatin regions associate with the nuclear periphery (Li *et al.* 2005). To determine whether ectopic CENP-A^{cnp1}-GFP chromatin overlaps with heterochromatin, we overexpressed CENP-A^{cnp1}-GFP at the intermediate level in a strain expressing mCherry-Swi6 at the endogenous level. We found a substantial overlap between CENP-A^{cnp1}-GFP and mCherry-Swi6 signals (Figure 1D), indicating that ectopic CENP-A^{cnp1}-GFP chromatin can assemble near or within heterochromatin at the nuclear periphery.

In *Drosophila*, the CENP-A homolog CID preferentially assembles at heterochromatin boundaries when it is overexpressed (Olszak *et al.* 2011). To investigate whether this is

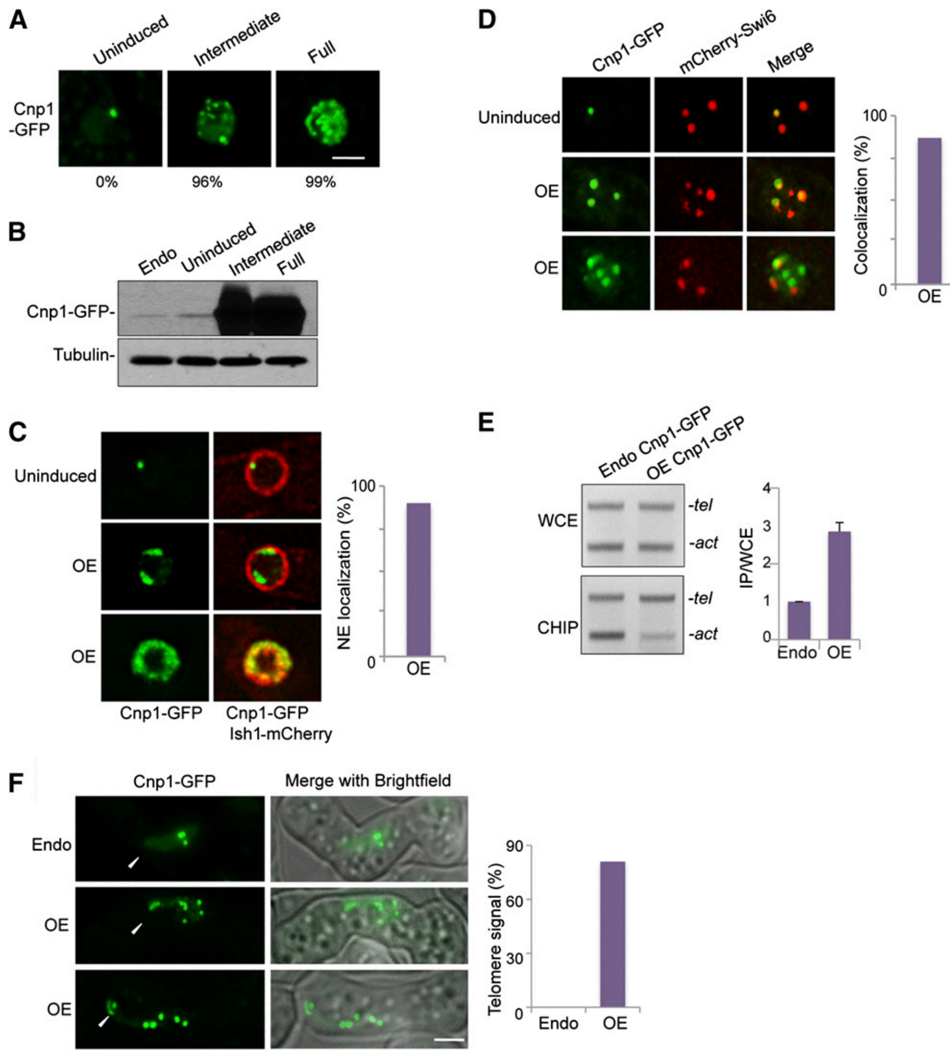

Figure 1 Overexpression of CENP-A^{cnp1} results in assembly of ectopic CENP-A^{cnp1} chromatin during mitosis and meiosis. (A) Cells overexpressing CENP-A^{cnp1}-GFP show excessive fluorescence foci in the nucleus. Wild-type cells carrying *nmt1*-CENP-A^{cnp1}-GFP were either uninduced (left), induced to an intermediate level (middle), or fully induced (right) by supplementation with 100, 0.05, or 0 μM of thiamine, respectively. The percentage of cells containing multiple CENP-A^{cnp1}-GFP foci (more than two) is indicated at the bottom. Note: due to low leaky expression of the *nmt1* promoter, uninduced cells show a single fluorescent focus in the nucleus that resembles cells expressing CENP-A^{cnp1}-GFP at the endogenous level. (B) Western blot analysis of total cell extracts from cells expressing CENP-A^{cnp1}-GFP at the endogenous level (endo) and cells carrying *nmt1*-CENP-A^{cnp1}-GFP that are uninduced, intermediately induced, or fully induced. Whole-cell extracts were subjected to immunoblotting with anti-GFP antibody. Tubulin was used as a loading control. (C) CENP-A^{Cnp1}-GFP overexpression in mitotic cells results in the assembly of ectopic CENP-A^{Cnp1} preferentially at the nuclear periphery. The nuclear envelope was visualized by the mCherry-tagged nuclear envelope protein Ish1. (Right) The percentage of intermediately induced cells showing nuclear periphery localization of CENP-A^{Cnp1}-GFP. (D) Ectopic CENP-A^{cnp1}-GFP preferentially assembles near heterochromatic regions. mCherry-Swi6 was used to visualize heterochromatin. Note: some of the excess CENP-A^{cnp1}-GFP foci overlap with the mCherry-Swi6 foci, indicating that ectopically assembled CENP-A chromatin preferentially forms within or near heterochromatin. (Right) The percentage of cells showing overlapping of mCherry-Swi6 and CENP-A^{Cnp1}-GFP. (E) Ectopic CENP-A^{cnp1}-GFP is enriched at heterochromatin boundaries. ChIP assays were performed using an antibody against GFP. Immunoprecipitated DNA was analyzed by competitive PCR with primers specific for a subtelomeric region (*tel*) and a control gene, *act1*$^+$ (*act*). Three independent experiments were performed. (Right) The relative fold enrichment was calculated by comparing the ratios of CENP-A^{cnp1}-GFP signals to control signals between ChIP and WCE fractions. The value for wild type was normalized to 1.0. WCE, whole-cell extracts. (F) Overexpression of CENP-A^{cnp1}-GFP results in ectopic assembly of CENP-A^{cnp1}-GFP near heterochromatin in meiotic cells. In control crosses from cells expressing CENP-A^{cnp1}-GFP at the endogenous level, 2–3 GFP foci, representing centromeres, form at the distal end of the elongated nucleus during the horsetail stage (top). At the leading edge of the horsetail, where telomeres cluster, no fluorescent signal is detectable (white arrowheads) (top). In contrast, horsetail stage zygotes overexpressing CENP-A^{cnp1}-GFP (OE) show excessive fluorescent foci (8–15), which often localize within the vicinity of both telomeres and centromeres. (Right) The frequency of cells showing fluorescent foci near telomeres. Bars, 2 μm.

also the case in fission yeast, we analyzed cells overexpressing CENP-A^{cnp1}-GFP using ChIP along with primers specific to a subtelomeric region. We found that CENP-A^{cnp1}-GFP is enriched at the heterochromatin boundary region (Figure 1E). This is consistent with a previous finding (Castillo *et al.* 2013).

Ectopic CENP-A^{cnp1}-GFP chromatin preferentially assembles near heterochromatin in meiotic cells

We also examined whether CENP-A^{cnp1}-GFP assembles at noncentromeric chromatin in meiotic cells overexpressing CENP-A^{cnp1}-GFP. During meiotic prophase, the nucleus shows an elongated morphology called a "horsetail." At this stage, the telomeres cluster beneath the SPB at the leading edge of the moving nucleus, and the centromeres cluster at the opposite (distal) end of the nucleus (Chikashige *et al.* 1994). As expected, in cells expressing an endogenous level of CENP-A^{cnp1}-GFP, little fluorescent signal was observed at the leading edge of the horsetail nucleus where the telomeres cluster, whereas two to three foci appear at the distal end containing the centromeres (Figure 1F). In contrast, in 81% of meiotic cells overexpressing CENP-A^{cnp1}-GFP, the "telomeric" edge of the moving nucleus becomes populated with multiple fluorescent foci (Figure 1F). In addition, the number of fluorescent

foci at the distal end increases from 3, typical for wild-type cells, to 7–12 (Figure 1F), indicating that excessive CENP-A^{cnp1} also localizes near centromeric regions. This demonstrates that, as in mitotic cells, the overexpression of CENP-A^{cnp1}-GFP in meiotic cells induces the assembly of ectopic CENP-A^{cnp1} chromatin at noncentromeric chromatin positioned close to or within heterochromatin.

CENP-A^{cnp1} overexpression causes chromosome missegregation during mitosis

To investigate the effect of ectopic CENP-A chromatin on cell growth, serial dilutions of cells carrying CENP-A^{cnp1}-GFP were plated in minimal media without thiamine. While control cells plated in the presence of 100 μM thiamine grow indistinguishably from wild-type cells expressing endogenous CENP-A^{cnp1}, cells in thiamine-depleted media exhibited much slower growth (Figure 2A). This is consistent with a recent study (Castillo *et al.* 2013). As a control, we also performed similar growth assays with cells overexpressing GFP-Dos1. As shown in Figure 2A, GFP-Dos1 overexpression has little effect on cell growth.

To determine the effect of ectopic CENP-A^{cnp1} chromatin on chromosome segregation, DAPI stainings were used. We found that 37% of cells examined display uneven, lagging, or stretched chromosomes during mitosis (Figure 2B), compared to 1% of wild-type cells expressing endogenous CENP-A^{cnp1}. This mirrors the chromosome segregation defects observed in *Drosophila* cells overexpressing the CENP-A homolog CID (Heun *et al.* 2006). Furthermore, in cells overexpressing CENP-A^{cnp1}-GFP, the nucleus is visible because the fluorescent signal diffuses throughout the nucleoplasm. This allows us to determine that, in a significant number of cells overexpressing CENP-A^{cnp1}-GFP, the nucleus appears abnormal in size and shape, consistent with the DAPI staining (Figure S5). These defects in nuclear morphology are further confirmed by analyzing the cells carrying the nuclear envelope marker Ish1-mCherry (Figure 2C). These data suggest that the localization of CENP-A^{cnp1} at ectopic loci interferes with the proper chromosome segregation.

CENP-A^{cnp1} overexpression results in chromosome missegregation during meiosis

How meiosis is affected by the overexpression of CENP-A has not been studied. To investigate the impact of ectopic CENP-A chromatin on chromosome segregation during meiosis, haploid cells of both mating types exhibiting ectopic CENP-A^{cnp1}-GFP foci were crossed. Normal meiosis produces asci, each of which contains four round, similar-sized spores (Figure 2D). But 38% of asci analyzed from this cross contain an irregular number of spores (Figure 2D). Even in the asci containing a normal number of spores, careful examination revealed that the spores are often abnormal in size and shape. In these asci, the spores' nuclei are easy to visualize because the excess CENP-A^{cnp1}-GFP signal diffuses throughout the nucleus. We found that, consistent with the aberrant spore formation, the spores in these asci contain abnormal numbers of nuclei and that the morphology of the nuclei also often appears deformed (Figure 2E). We also examined the asci from the crosses between a strain carrying ectopic CENP-A^{cnp1}-GFP foci and a wild-type strain expressing CENP-A^{cnp1}-GFP at the endogenous level. These crosses also give rise to a significant number of asci with defective spores (16%). These observations indicate that ectopically assembled CENP-A also interferes with chromosome segregation during meiosis.

Ectopically localized CENP-A^{cnp1} can recruit kinetochore components

We next investigated whether ectopic CENP-A^{cnp1} chromatin can nucleate the assembly of kinetochores. In fission yeast, kinetochores consist of two distinct protein complexes, DASH and Ndc80-MIND-Spc7. Dad1 is a component of the DASH complex and exclusively localizes to centromeres (Liu *et al.* 2005; Sanchez-Perez *et al.* 2005). To determine whether Dad1 is recruited to sites of ectopically assembled CENP-A, we constructed a strain carrying *nmt1*-regulated CENP-A^{cnp1}-mCherry and Dad1-GFP under its native promoter. In cells containing uninduced CENP-A^{cnp1}-mCherry, Dad1-GFP and CENP-A^{cnp1}-mCherry colocalize at the centromeres, as expected (Figure 3A). In contrast, multiple Dad1-GFP spots form within the nucleus in 26% of the cells overexpressing CENP-A^{cnp1}-mCherry that were analyzed (Figure 3A). These Dad1-GFP spots also overlap with excess CENP-A^{cnp1}-mCherry foci (Figure 3A). This indicates that Dad1-GFP is effectively recruited to sites containing ectopically assembled CENP-A^{cnp1}-mCherry. However, not all of the ectopic CENP-A^{cnp1}-mCherry foci overlap with the Dad1-GFP signals, suggesting that only a subset of the regions containing ectopic CENP-A chromatin are able to recruit kinetochore components. A similar phenomenon was identified in *Drosophila* (Heun *et al.* 2006).

To investigate whether components of the Ndc80-MIND-Spc7 kinetochore complex can also be recruited to ectopic CENP-A sites, we overexpressed CENP-A^{cnp1}-mCherry in cells expressing GFP-tagged Ndc80 from its native promoter. Same as Dad1-GFP, Ndc80-GFP specifically associates with centromeres and forms a single focus in wild-type cells. In contrast, multiple nuclear Ndc80-GFP foci form in 21% of the cells overexpressing CENP-A^{cnp1}-mCherry that were analyzed. Furthermore, these foci overlap with the ectopic CENP-A^{cnp1}-mCherry chromatin (Figure 3B). These results further indicate that a subset of ectopic CENP-A chromatin sites can recruit kinetochore components.

CENP-A^{cnp1} overexpression causes spindle disorganization during mitosis

We next examined spindle organization in mitotic cells overexpressing CENP-A^{cnp1}. For this purpose, we engineered a strain expressing GFP-labeled tubulin, Tub1-GFP, under its native promoter, and CENP-A^{cnp1}-TAP under the *nmt1* promoter. Replacing endogenous CENP-A^{cnp1} by CENP-A^{cnp1}-TAP

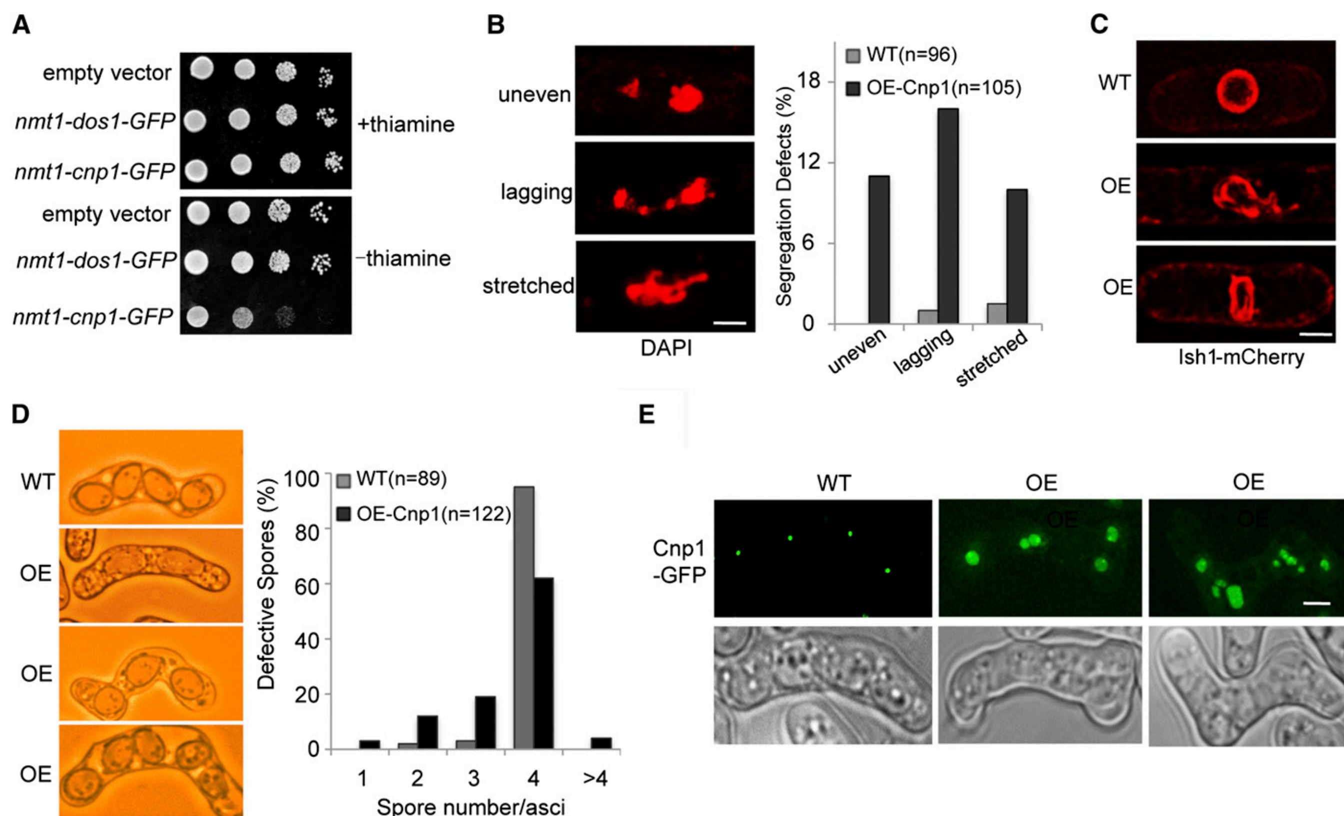

Figure 2 Overexpression of CENP-A^{cnp1} results in severe chromosome segregation defects during mitosis and meiosis. (A) Overexpression of CENP -A^{cnp1} causes severe growth defects. Cells harboring *nmt1*-CENP-A^{cnp1}-GFP were grown in minimal medium with thiamine to suppress its expression (top) or lacking thiamine to induce overexpression of CENP-A^{cnp1}-GFP (bottom). Wild-type cells carrying an empty vector or *nmt1-dos1*-GFP were used as a control. (B) Overexpression of CENP-A^{cnp1}-GFP causes chromosome segregation defects in mitotic cells, as shown by DAPI staining. (Right) The percentage of mitotic cells showing uneven, lagging, or stretched chromosomes. (C) Nuclear morphology defects in cells overexpressing CENP-A^{cnp1}-GFP were visualized by Ish1-mCherry signal. WT, wild-type cells expressing CENP-A^{cnp1}-GFP at the endogenous level. (D) Overexpression of CENP-A^{cnp1}-GFP causes abnormal spore formation. Haploid cells of both mating types carrying *nmt1*-CENP-A^{cnp1}-GFP were sporulated after CENP-A^{cnp1}-GFP expression was fully induced. (Right) The frequency distribution of the number of spores per ascus. Crosses between wild-type cells expressing CENP-A^{cnp1}-GFP at the endogenous level (WT) were used as a control. (E) Asci from strains overexpressing CENP-A^{cnp1}-GFP display an aberrant number of nuclei. The nuclei could be visualized due to diffusion of the CENP-A^{cnp1}-GFP signal throughout the nucleus. Bars, 2 μm.

under its native promoter does not affect growth (Figure S2), indicating that TAP-tagged CENP-A^{cnp1} is functional. During mitosis, sister chromatids are pulled to opposite poles of the nucleus by a straight microtubule spindle extending between two SPBs (Hiraoka *et al.* 1984). Strikingly, in 39% of mitotic cells overexpressing CENP-A^{cnp1}-TAP, multiple small Tub1-GFP filaments are observed in the nucleus (Figure 3C), indicating that the spindle is highly disorganized during the stage. Interestingly, these small spindles are primarily localized at the nuclear periphery (Figure 3C), resembling the nuclear periphery distribution of ectopic CENP-A^{cnp1}-GFP. In a complementary experiment, we overexpressed CENP-A^{cnp1}-mCherry in wild-type cells expressing Tub1-GFP. The multiple small Tub1-GFP filaments were also observed in these cells. This experiment also allowed us to determine that many, although not all, of the Tub1-GFP filaments overlap with ectopic CENP-A^{cnp1}-mCherry foci (Figure 3D). These data indicate that overexpression of CENP-A^{cnp1} causes spindle disorganization during mitosis and suggest that the abnormal spindle fragments may associate with some ectopically assembled CENP-A^{cnp1}-mCherry chromatin.

Ectopic CENP-A chromatin persists through multiple generations in the absence of CENP-A overexpression

To determine whether ectopically localized CENP-A^{cnp1} can be maintained through generations, we carried out the pulse induction of CENP-A^{cnp1}-GFP overexpression assay in wild-type cells using *nmt1*-CENP-A^{cnp1}-GFP. After ectopic CENP-A^{cnp1}-GFP foci were induced, we stopped the overexpression by adding thiamine. The repression of the *nmt1* promoter expression can be achieved in less than one cell cycle (2–3 hr) (Maundrell 1990). Ectopic CENP-A^{cnp1}-GFP foci were then monitored by fluorescence microscopy for 24 hr. Our Western blot analysis shows that after a 24-hr repression of the *nmt1* promoter, the CENP-A^{cnp1}-GFP level returned to its level before pulse induction (Figure 4A). After pulse induction of CENP-A^{cnp1}-GFP overexpression, ~40% of cells exhibiting ectopic CENP-A can still divide for several generations with a doubling time of 3–4 hr. We found that after 24 hr of growth in the

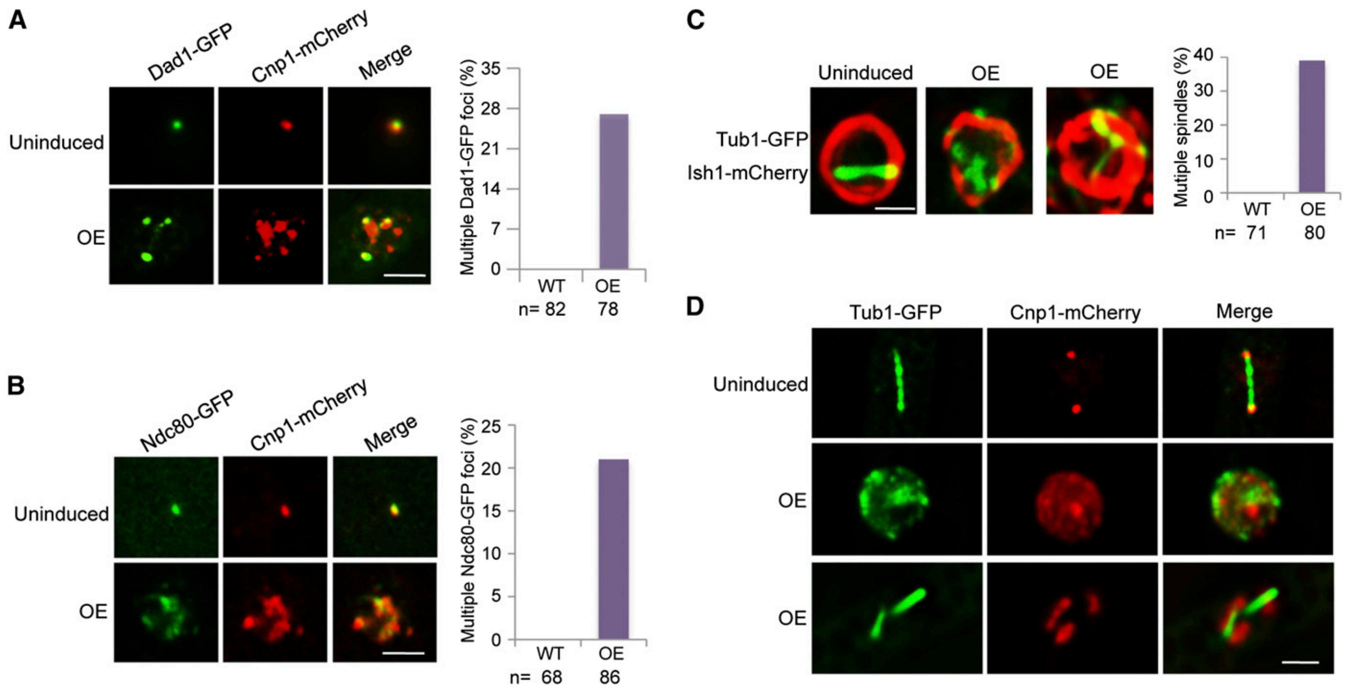

Figure 3 Overexpression of CENP-A^{cnp1}-GFP causes ectopic recruitment of kinetochore components and spindle disorganization. (A) Overexpression of CENP-A^{cnp1} results in aberrant localization of the kinetochore protein Dad1. Cells carrying Dad1-GFP under its native promoter and *nmt1*-CENP-A^{cnp1}-mCherry were used. In uninduced cells, a single nuclear CENP-A^{cnp1}-mCherry focus was observed, and the focus colocalizes with Dad1-GFP (top). In induced cells, multiple Dad1-GFP foci form and they colocalize with ectopic CENP-A^{cnp1}-mCherry (bottom). (Right) The frequency of cells showing aberrant localization of Dad1-GFP in uninduced and induced cells. (B) Cells with ectopically assembled CENP-A^{cnp1} show abnormal localization of the kinetochore protein Ndc80. Cells containing Ndc80-GFP under the endogenous promoter and *nmt1*-CENP-A^{cnp1}-mCherry were used. In uninduced cells, a single CENP-A^{cnp1}-mCherry spot that colocalizes with Ndc80-GFP forms. Upon CENP-A^{cnp1} induction, multiple Ndc80-GFP foci form and they colocalize with ectopic CENP-A^{cnp1}-mCherry. (Right) The frequency of cells showing aberrant localization of Ndc80-GFP in uninduced and induced cells . (C) CENP-A^{cnp1} mislocalization causes abnormal spindle microtubule distribution. CENP-A^{cnp1} was overexpressed in cells expressing GFP-labeled α-tubulin (Tub1-GFP) at the endogenous level. Ish1-mCherry was used to visualize the nucleus. In uninduced mitotic cells, a single straight spindle bundle was visible, which extends between two oppositely positioned SPBs embedded in the nuclear envelope. In induced mitotic cells (OE), multiple microtubule filaments were observed in disarray, most frequently near the nuclear periphery. (Right) The frequency of uninduced and induced cells showing nuclear microtubule filaments. (D) Multiple microtubules attach to sites of ectopic CENP-A^{cnp1} assembly. Wild-type cells containing *nmt1*-CENP-A^{cnp1}-mCherry and expressing endogenous Tub1-GFP were used. In uninduced mitotic cells, centromeres, visualized by endogenous CENP-A^{cnp1}-mCherry, are pulled toward opposite poles of the nucleus by a straight microtubule spindle, which is visualized by Tub1-GFP (top). In induced mitotic cells, multiple microtubule bundles are observed, which often colocalize with ectopic CENP-A^{cnp1}-mCherry sites. These observations suggest that microtubules in cells overexpressing CENP-A^{cnp1}-mCherry attach to noncentromeric regions. Bars: 2 μm (A and B); 1 μm (C and D).

thiamine-containing medium, multiple CENP-A^{cnp1}-GFP foci could still be observed in 62% of the cells (Figure 4B). The enrichment of the signal at the heterochromatin boundary at this stage was further confirmed by ChIP (Figure S6). In fact, even after growing the cells in rich medium for several days, we could still find cells that contained several CENP-A^{cnp1}-GFP foci. These results indicate that ectopic CENP-A can persist for multiple generations in the absence of CENP-A overexpression. In fission yeast, after mitosis, cells progress quickly into S phase, during which a septum grows across the center of cells and divides old cells into two new daughter cells. We noted that the number and the distribution pattern of excessive CENP-A^{cnp1}-GFP foci within the nucleus of the two daughter cells are almost identical (Figure S7). This finding supports the view that ectopically assembled CENP-A in fission yeast can be maintained throughout the cell cycle.

We also conducted pulse overexpression of CENP-A^{cnp1}-mCherry in cells expressing CENP-A^{cnp1}-GFP at endogenous level. In uninduced cells, CENP-A^{cnp1}-mCherry forms a single red fluorescent focus at the centromere that colocalizes with endogenous CENP-A^{cnp1}-GFP, as expected (Figure 4C). Following pulse induction, CENP-A^{cnp1}-mCherry effectively assembles outside of centromeres to form excessive foci. Multiple green CENP-A^{cnp1}-GFP signals were also observed in 32% of the cells analyzed (Figure 4C). Importantly, these CENP-A^{cnp1}-GFP foci overlapped with the ectopic CENP-A^{cnp1}-mCherry foci, and this colocalization persisted for several generations. These data indicate that endogenous CENP-A^{cnp1}-GFP can be recruited *de novo* to ectopic CENP-A^{cnp1}-mCherry sites and provide further evidence for the inheritance of ectopic CENP-A chromatin.

CATD domain of CENP-A^{cnp1} is required for the assembly of CENP-A^{cnp1} at ectopic sites

The CATD domain is known to be required for targeting CENP-A to endogenous centromeres (Black *et al.* 2004, 2007; Fachinetti

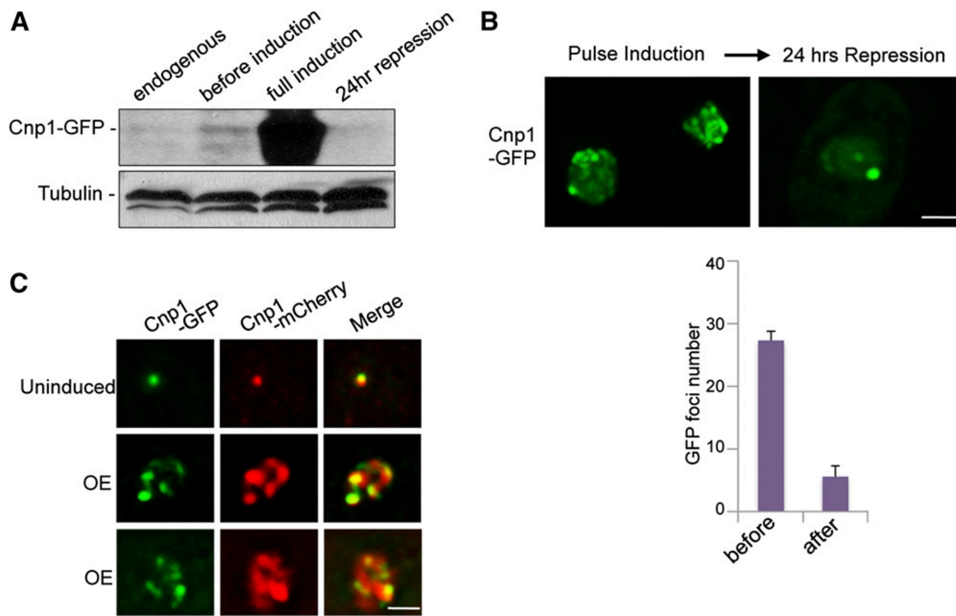

Figure 4 Ectopic CENP-A chromatin persists for multiple generations in the absence of CENP-A overexpression. (A) Extracts from cells carrying *nmt1*-CENP-A^{cnp1}-GFP that are uninduced, fully induced, or repressed for 24 hr following full induction were analyzed by Western blotting with an anti-GFP antibody. Cells expressing CENP-A^{cnp1}-GFP at the endogenous level were used as a control. (B) Ectopic CENP-A^{cnp1}-GFP foci are maintained for multiple generations following pulse overexpression of CENP-A^{cnp1}-GFP. Cells carrying *nmt1*-CENP-A^{cnp1}-GFP were incubated for 24 hr in minimal medium without thiamine to induce the ectopic assembly of CENP-A^{cnp1} before addition of thiamine to repress the overexpression. The distribution pattern of CENP-A^{cnp1}-GFP was then monitored by fluorescence microscopy for 24 hr. (Bottom) The average number of GFP foci before and after repression. (C) Ectopically assembled CENP-A^{cnp1} can recruit CENP-A^{cnp1} *de novo*. Cells carrying both CENP-A^{cnp1}-GFP under its native promoter and *nmt1*-CENP-A^{cnp1}-mCherry were used. Uninduced cells show both fluorescent proteins exclusively at centromeres. Following pulse overexpression of CENP-A^{cnp1}-mCherry, multiple CENP-A^{cnp1}-GFP that colocalized with the ectopic CENP-A^{cnp1}-mCherry foci was observed, and this colocalization persisted for several generations after pulse overexpression. Bars: 2 μm.

et al. 2013). To investigate the role of the CATD domain in the assembly of ectopic CENP-A chromatin, we created a CATD-deleted version of CENP-A^{cnp1}, Cnp1$^{CATD\Delta}$ (Figure 5A), which was constructed with a GFP tag under the *nmt1* promoter. Under an uninduced condition, as expected, this mutant protein fails to localize to the centromere (Figure 5B). When overexpressed, the mutant protein also fails to assemble at noncentromeric chromatin and instead appears diffused throughout the nucleus (Figure 5B). Our Western blot analysis also indicates that the mutant protein expresses at a similar level to full-length CENP-A^{cnp1}-GFP in both induced and uninduced cells (Figure S8). To determine whether Cnp1$^{CATD\Delta}$ -GFP is chromatin-bound, we performed *in situ* chromatin-binding assays. We found that Cnp1$^{CATD\Delta}$-GFP can be effectively washed away from the nucleus by Triton X-100 (Figure S9), indicating that Cnp1$^{CATD\Delta}$-GFP does not assemble stably with chromatin. Consistent with this, overexpression of the mutant protein had little effect on cell growth (Figure 5C). Together, these data indicate that the CATD domain is also required for targeting CENP-A to noncentromeric chromatin and further demonstrate that the growth defect in cells overexpressing full-length CENP-A is a direct consequence of CENP-A assembly at ectopic loci.

N-terminal domain of CENP-A^{cnp1} is important for preventing assembly of CENP-A at ectopic loci

To examine the role of the N-terminal domain of CENP-A in the assembly of CENP-A at ectopic loci, we constructed a wild-type strain carrying a N-terminal domain-deleted CENP-A^{cnp1} (Cnp1$^{N\Delta}$) under the *nmt1* promoter (Figure 5A). Surprisingly, 41% of the cells carrying *nmt1*-Cnp1$^{N\Delta}$-GFP under an uninduced condition show excessive GFP spots, most of which concentrate at the nuclear periphery (Figure 6A). This differed sharply from what was observed in cells expressing full-length CENP-A^{cnp1}-GFP under the same condition (Figure 6A), indicating that deletion of the N-terminal domain of CENP-A^{cnp1} results in the ectopic assembly of CENP-A at noncentromeric regions. We also examined the average number of fluorescent foci in cells overexpressing either CENP-A^{cnp1}-GFP or Cnp1$^{N\Delta}$-GFP at the intermediate level, under which discernible GFP foci formation occurs. Under this condition, cells overexpressing Cnp1$^{N\Delta}$-GFP exhibit an approximately threefold increase in fluorescent foci compared with cells overexpressing CENP-A^{cnp1}-GFP (Figure 6B). Furthermore, cells overexpressing Cnp1$^{N\Delta}$ exhibit significantly slower growth than cells overexpressing CENP-A^{cnp1} (Figure 6C). The severe growth defect associated with the overexpression of Cnp1$^{N\Delta}$ is likely the result of aggravated chromosome missegregation due to more widespread ectopic Cnp1$^{N\Delta}$ assembly. Together, these observations suggest that the N-terminal domain of CENP-A plays a role in preventing the assembly of CENP-A at noncentromeric chromatin.

N-terminal domain of CENP-A^{cnp1} mediates ubiquitin-dependent proteolysis of CENP-A

Previous studies have shown that ubiquitin-mediated proteolysis of CENP-A is important for preventing CENP-A from euchromatin localization in budding yeast and *Drosophila* (Collins *et al.* 2004; Moreno-Moreno *et al.* 2006). To determine whether the N-terminal domain of CENP-A^{cnp1} plays a role in targeting the protein for degradation via the ubiquitin pathway in fission yeast, we first examined the stability of N-terminal-deleted CENP-A^{cnp1}-GFP compared to wild-type CENP-A^{cnp1}-GFP. Western blot analysis reveals that,

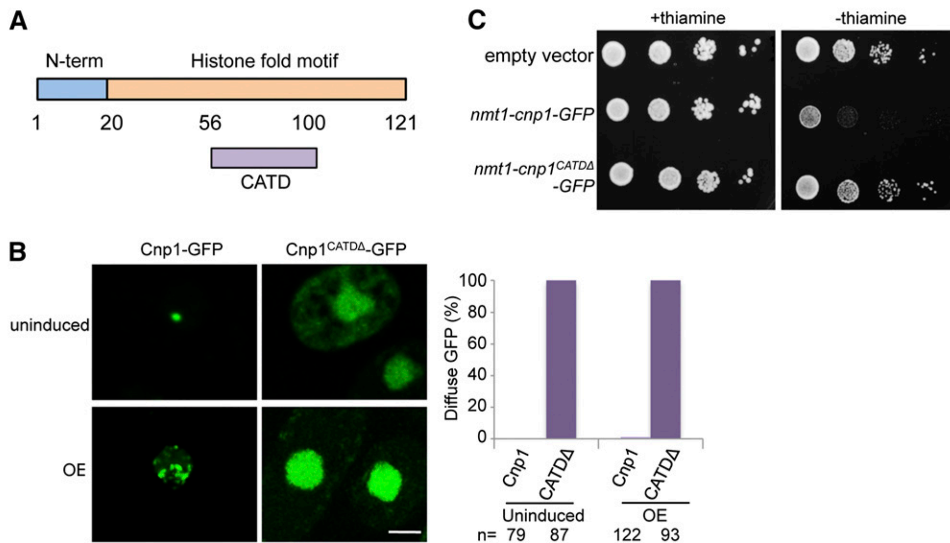

Figure 5 The CATD domain of CENP-A is required for recruitment of CENP-A^{cnp1} to ectopic loci. (A) Schematic representation of CENP-A^{cnp1} domain structures. N-term, N terminus. (B) Cells carrying GFP-labeled, CATD-deleted CENP-A^{cnp1} (Cnp1$^{CATD\Delta}$-GFP) under the *nmt1* promoter exhibit diffuse GFP signal throughout the nucleus under both uninduced and induced conditions. (Right) The average number of cells showing diffused Cnp1$^{CATD\Delta}$-GFP in the nucleus. Bar, 2 μm. (C) Overexpression of Cnp1$^{CATD\Delta}$ does not affect cell growth. Serial dilutions of wild-type cells carrying empty vector, CENP-A^{cnp1}, and Cnp1$^{CATD\Delta}$-GFP under the *nmt1* promoter were grown on minimal medium under either uninduced or induced conditions.

while most wild-type CENP-A^{cnp1}-GFP is degraded within 2 hr of treatment with cycloheximide, a substantial amount of N-terminally deleted CENP-A^{cnp1} remains present even 3 hr post-treatment with the drug (Figure 6D). These data indicate that deleting the N-terminal domain of CENP-A^{cnp1} increases the stability of the protein. We next investigated whether the N terminus of CENP-A^{cnp1} is required for ubiquitination of CENP-A^{cnp1}. For this purpose, we performed affinity pulldown using TUBEs with strains expressing CENP-A^{cnp1}-GFP or Cnp1$^{N\Delta}$-GFP. We observed a laddering pattern for full-length CENP-A^{cnp1} after immunoprecipitation with agarose–TUBEs. In contrast, the control sample immunoprecipitated with agarose lacking TUBEs does not show the laddering pattern (Figure 6E), indicating that CENP-A^{cnp1} is polyubiquitinated. A similar laddering pattern is also observed for N-terminally deleted CENP-A^{cnp1}, but the level is significantly reduced (Figure 6E). These data demonstrate that the N terminus of CENP-A is required for CENP-A^{cnp1} ubiquitination and further suggest that the N-terminal domain plays a role in preventing the assembly of CENP-A chromatin at noncentromeric regions by targeting CENP-A for degradation via the ubiquitin-dependent pathway.

Ectopic CENP-A chromatin assembly is suppressed by the overexpression of histone H3 or H4

Previous work indicates that the relative ratio of histone H3 to histone H4 influences the incorporation of CENP-A^{cnp1} at endogenous centromeres (Castillo *et al.* 2007). To investigate the influence of H4 on the assembly of CENP-A^{cnp1} at noncentromeric chromatin, we overexpressed H4 from the *nmt1* promoter in wild-type cells also overexpressing CENP-A^{cnp1}-GFP. We found that the number of ectopic CENP-A^{cnp1} foci is significantly reduced in these cells (Figure 7A). Western blot analysis indicates that the level of CENP-A^{cnp1}-GFP in these cells remains similar to that of cells overexpressing CENP-A^{cnp1}-GFP alone (Figure 7B). This indicates that the reduction in ectopic CENP-A^{cnp1} foci observed in these cells is not the result of a reduction in the amount of CENP-A^{cnp1}-GFP. Furthermore, overexpressing H4 is able to rescue the growth defect associated with the overexpression of CENP-A^{cnp1}-GFP (Figure 7C). We next examined how overexpression of histone H3 affects the assembly of CENP-A^{cnp1}-GFP at ectopic loci. We found that, similar to overexpressing H4, H3 overexpression results in a reduction in the number of ectopic CENP-A^{cnp1}-GFP foci in cells overexpressing CENP-A^{cnp1}, although to a lesser extent (Figure 7D). In these cells, the level of CENP-A^{Cnp1}-GFP also remains similar to that of cells overexpressing CENP-A^{Cnp1}-GFP alone (Figure 7B). Intriguingly, unlike H4 overexpression, H3 overexpression is unable to rescue the slow growth caused by ectopic CENP-A^{cnp1} and, instead, exacerbated the growth defect (Figure 7E). In a previous study, excess H3, but not H4, was shown to interfere with the loading of CENP-A^{cnp1} at endogenous centromeres, which in turn interferes with kinetochore assembly and leads to chromosome missegregation (Castillo *et al.* 2007). This likely explains why overexpression of H3, although resulting in reduction of ectopic CENP-A^{cnp1}, cannot rescue the growth defects caused by mislocalized CENP-A^{cnp1}.

Discussion

How centromere position is specified and how noncentromeric chromatin is protected from assembling unwanted centromeres are central questions in chromatin regulation. Here we demonstrate that, consistent with previous findings (Choi *et al.* 2012; Castillo *et al.* 2013), overexpression of CENP-A^{cnp1} in fission yeast results in the assembly of CENP-A^{cnp1} at noncentromeric chromatin and in growth defects. Similar observations have been reported in *Drosophila* (Heun *et al.* 2006). We further show that, also similar to ectopic CID in *Drosophila*, ectopically assembled CENP-A^{Cnp1} in fission yeast is also able to recruit kinetochore components. We also demonstrate that coincident with the ectopic localization of CENP-A^{Cnp1} is the disorganization of spindle

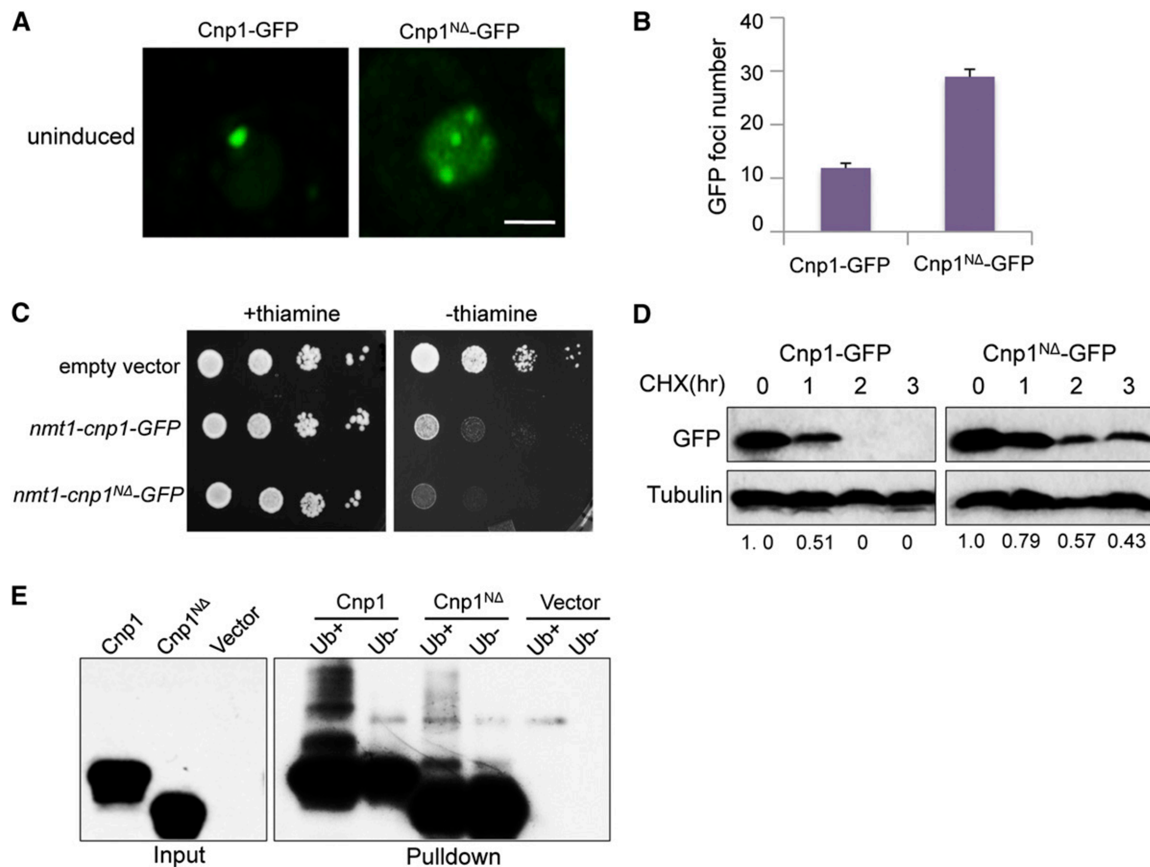

Figure 6 The N-terminal domain of CENP-A is required for preventing assembly of CENP-A^{cnp1} at ectopic loci. (A) Cells containing N-terminus-deleted CENP-A^{cnp1}-GFP (Cnp1$^{N\Delta}$-GFP) under the *nmt1* promoter display multiple GFP foci under uninduced condition. This contrasts with the single focus present in cells expressing full-length CENP-A-GFP under the same condition. Bar, 2 μm. (B) Quantification of GFP foci in cells overexpressing Cnp1$^{N\Delta}$-GFP or CENP-A^{cnp1}-GFP at intermediate level. Cells overexpressing of Cnp1$^{N\Delta}$-GFP show a higher number of nuclear GFP foci than control cells expressing CENP-A^{cnp1}-GFP. (C) Cells overexpressing Cnp1$^{N\Delta}$-GFP show slower growth than cells overexpressing CENP-A^{cnp1}-GFP. Serial dilutions of indicated strains were plated in minimal medium with or without thiamine. (D) Deletion of the N-terminal domain of CENP-A^{cnp1} increases the stability of the protein. Cells expressing Cnp1$^{N\Delta}$-GFP or CENP-A^{cnp1}-GFP under uninduced condition were treated with cycloheximide. Lysates from cells collected at indicated time points (hr) following treatment with cycloheximide were analyzed by Western blotting. Tubulin was used as a loading control. (E) The N-terminal domain of CENP-A^{cnp1} is required for ubiquitination of the protein. Extracts from cells overexpressing full-length or N-terminus-deleted CENP-A^{cnp1}-GFP were subjected to precipitation with TUBEs (Ub$^+$) or control beads (Ub$^-$) (LifeSensors, Inc.). Precipitates were analyzed by Western blotting using an anti-GFP antibody.

microtubules. Our data suggest that spindle microtubules may be attaching to noncentromeric sites where CENP-A^{cnp1} is assembled. This is consistent with the presence of lagging, or stretched, chromosomes in these cells, which can result from the various pulling forces along a chromosome exerted by the ectopically localized microtubules. Importantly, we provide evidence that, in meiotic cells, an overabundance of CENP-A^{Cnp1}-GFP also results in the assembly of CENP-A at non-centromeric chromatin and concomitant chromosome segregation defects. To our knowledge, this study is the first to report these phenomena in meiotic cells. Our findings suggest that an abnormally high level of CENP-A can induce the assembly of ectopic centromeres in both mitotic and meiotic cells.

A key attribute of an epigenetic mark is its heritability. In *Drosophila*, ectopically assembled CID induced by its over-expression can self-propagate through the cell cycle (Olszak *et al.* 2011). Here, we provide the first evidence for a similar phenomenon in fission yeast. Cells induced to assemble CENP-A^{Cnp1}-GFP at ectopic loci through pulse overexpression display ectopic fluorescent foci even up to 3 days after terminating the overexpression of CENP-A^{Cnp1}-GFP, indicating that ectopically assembled CENP-A^{cnp1} chromatin persists through multiple generations. Consistent with this, endogenous CENP-A^{cnp1}-GFP can be recruited *de novo* to ectopic CENP-A^{cnp1}-mCherry sites, and the ectopic GFP foci persist for several generations in the absence of CENP-A^{cnp1}-mCherry overexpression. These results support CENP-A^{cnp1} as the epigenetic mark that defines centromeres, as well as the conserved epigenetic nature of regional centromeres.

In budding yeast, overexpression of the CENP-A homolog Cse4 can lead to its promiscuous incorporation at non-centromeric regions (Henikoff and Furuyama 2012; Krassovsky *et al.* 2012). Although recent work reported that the non-centromeric Cse4 in budding yeast cells is able to recruit

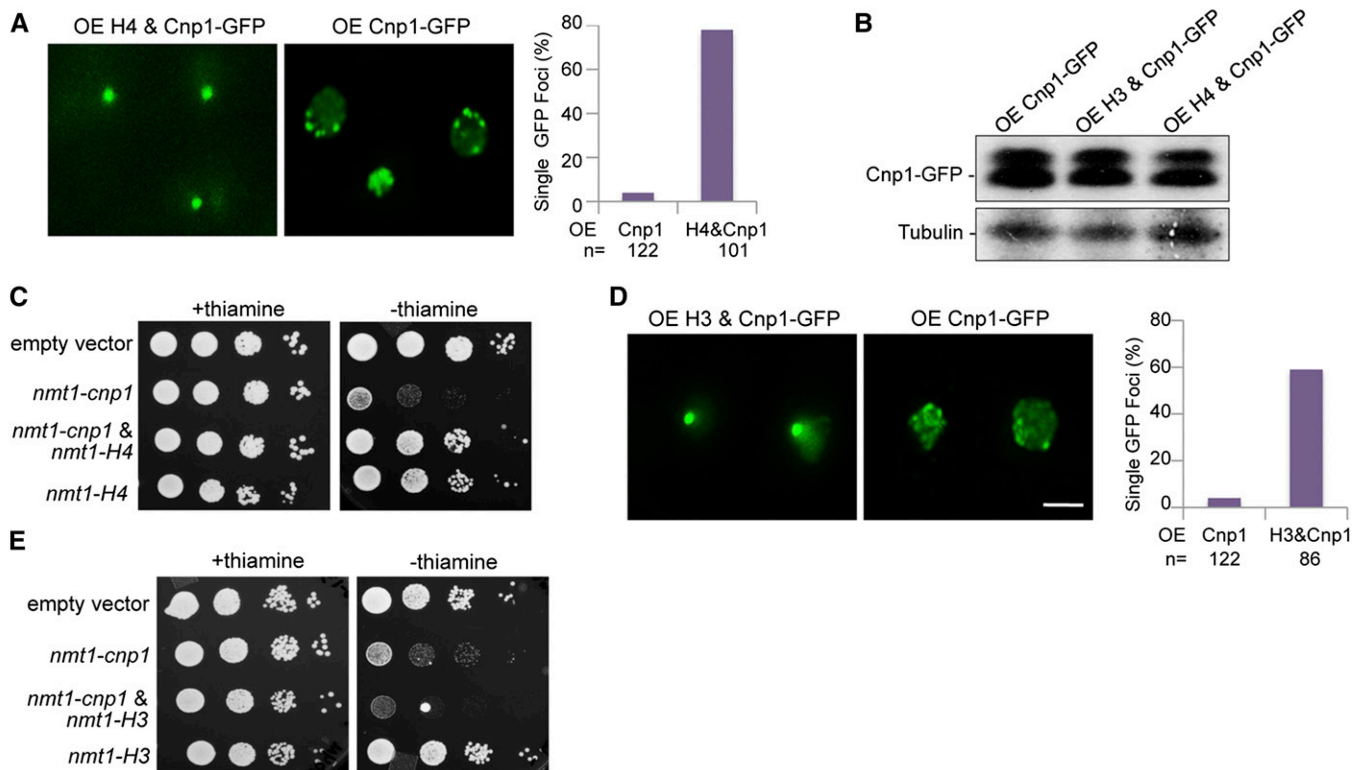

Figure 7 Overexpression of histone H3 or H4 affects the assembly of CENP-A at ectopic loci. (A) Overexpressing histone H4 results in a reduction of ectopic CENP-A^{cnp1}-GFP foci. Cells carrying *nmt1*-CENP-A^{cnp1}-GFP and H4 under the *nmt1* promoter were incubated for 24 hr in minimal media without thiamine (left). Cells overexpressing CENP-A^{cnp1}-GFP alone were used as a control; the frequency of cells displaying a single fluorescent focus is indicated (right). (B) Western blot analysis of extracts from cells overexpressing both histone H3 and CENP-A^{cnp1}-GFP or both histone H4 and CENP-A^{cnp1}-GFP. Cells overexpressing CENP-A^{cnp1}-GFP alone were used as a control. The lysates were immunoblotted with an anti-GFP antibody. Tubulin was used as a loading control. (C) Overexpression of histone H4 rescues the growth defects associated with overexpression of CENP-A^{cnp1}-GFP. Serial dilutions of indicated strains were grown on minimal medium with or without thiamine. (D) Overexpression of histone H3 causes a decrease in ectopic CENP-A^{cnp1}-GFP foci. (Right) The frequency of cells displaying a single fluorescent focus. (E) Overexpression of histone H3 aggravates the growth defect associated with overexpression of CENP-A^{cnp1}-GFP. Serial dilutions of indicated strains were plated onto minimal medium with or without thiamine. Bars, 2 μm.

kinetochore components (Lefrancois *et al.* 2013), overexpression of Cse4 in budding yeast does not affect growth or chromosome segregation, indicating that functional neocentromeres do not appear to form in these cells (Collins *et al.* 2004; Crotti and Basrai 2004; Henikoff and Furuyama 2012). Ubiquitin-dependent proteolysis has been shown to restrict Cse4 to native centromeres by tightly regulating Cse4 levels (Collins *et al.* 2004; Hewawasam *et al.* 2010; Ranjitkar *et al.* 2010). It is conceivable that, in budding yeast, stable assembly of Cse4 at ectopic loci, and consequent ectopic centromere formation, are effectively inhibited by rapid degradation of mislocalized Cse4 via the ubiquitin-dependent pathway. Alternatively, the nonepigenetic nature of centromere assembly in budding yeast may act to inhibit neocentromere assembly even in the presence of excess Cse4.

In this study, we provide evidence that, in vegetative cells, ectopic CENP-A^{cnp1} preferentially assembles at the nuclear periphery, especially near heterochromatin. This is consistent with previous findings in *Drosophila* and fission yeast (Olszak *et al.* 2011; Castillo *et al.* 2013). Importantly, we provide the first evidence that ectopic CENP-A^{cnp1} chromatin also preferentially assembles near heterochromatin in meiotic cells. Our data highlight the notion that the chromatin state between the heterochromatin–euchromatin boundary is favored for the formation of ectopic CENP-A chromatin, possibly due to its resemblance to the native regional centromere, which is embedded within heterochromatin. Consistent with this notion, studies in fission yeast and *Neurospora crassa* have shown that peri-centromeric heterochromatin plays an important role in the assembly of CENP-A at native centromeres (Kagansky *et al.* 2009; Smith *et al.* 2011). Furthermore, disruption of heterochromatin interferes with the assembly of CENP-A chromatin at ectopic sites in *Drosophila* (Olszak *et al.* 2011).

The role of the structural domains of CENP-A in ectopic assembly of CENP-A chromatin has not been previously explored. In this study, we assessed how the CATD and N-terminal domains of CENP-A^{cnp1} affect the assembly of ectopic CENP-A chromatin. The CATD domain, which resides within the histone fold domain of CENP-A at its C terminus, is essential for targeting the protein to centromeres (Black *et al.* 2004, 2007; Fachinetti *et al.* 2013). Our findings indicate that the CATD domain is also required for the

recruitment of CENP-A^{cnp1} to ectopic loci. At native centromeres, CENP-A interacts with H4 through the CATD domain to form a CENPA-H4 dimer. CENPA-H4 dimers, together with histones H2A and H2B, are assembled into centromeric nucleosomes that replace canonical H3-containing nucleosomes at centromeric regions (Sekulic *et al.* 2010; Bassett *et al.* 2012). It is conceivable that ectopically assembled CENP-A nucleosomes form through a similar mechanism requiring interaction between the CATD domain of CENP-A and H4. The CATD domain of CENP-A is also known to directly interact with HJURP and CENP-N, and these interactions appear to be important for the localization of CENP-A at centromeres (Carroll *et al.* 2009; Foltz *et al.* 2009; Bernad *et al.* 2011). It would be interesting to determine whether HJURP and CENP-N also interact with ectopically assembled CENP-A through its CATD domain and whether these interactions are required for the *de novo* assembly of CENP-A at ectopic loci.

An intriguing finding from this study is that deletion of the N terminus of CENP-A^{cnp1} results in assembly of CENP-A at ectopic loci. This suggests that the N terminus of CENP-A^{cnp1} plays a role in inhibiting the assembly of CENP-A^{cnp1} at noncentromeric chromatin. Consistent with this, overexpression of N-terminally deleted CENP-A^{cnp1} retards growth more strongly than overexpression of wild-type CENP-A^{cnp1}. Our results further show that the N terminus of CENP-A^{cnp1} is required for polyubiquitination of CENP-A. In agreement with this, deletion of the domain enhances the stability of the protein. However, removing the N terminus does not completely abolish ubiquitination (Figure 6E), indicating that additional unknown mechanisms function to ubiquitinate CENP-A^{Cnp1}. This possibility is supported by our observation that removing the N-terminal domain does not completely stabilize the protein (Figure 6D). Together, these findings suggest that the N terminus of CENP-A^{Cnp1} contributes to the exclusion of CENP-A^{Cnp1} from noncentromeric chromatin by targeting mislocalized CENP-A^{Cnp1} for degradation via the ubiquitin-dependent pathway. This is consistent with a study showing that, in budding yeast, the N terminus of Cse4 mediates polyubiquitination of the protein (Au *et al.* 2013), although it remains unknown whether deleting the domain results in assembly of Cse4 at ectopic loci.

Here we also provide the first evidence that increasing the level of either histone H3 or H4 in cells overexpressing CENP-A^{cnp1} is able to inhibit the assembly of CENP-A^{cnp1} chromatin at ectopic loci. In the case of H4, its overexpression can rescue the growth and chromosome segregation defects caused by the overexpression of CENP-A^{cnp1}. Additional experiments are needed to elucidate the mechanisms by which this striking phenomenon takes place. One attractive hypothesis is that, in cells overexpressing CENP-A^{cnp1} alone, the interaction of excessive CENP-A^{cnp1} with histone H4 exhausts the pool of H4 available to assemble with histone H3. This leads to a significant reduction in H3-containing nucleosomes and, consequently, deposition of CENP-A nucleosomes at noncentromeric regions (Figure S10). However, when both CENP-A^{cnp1} and H4 are simultaneously overexpressed, sufficient H3–H4 dimers are assembled. Since noncentromeric regions preferentially assemble canonical H3 nucleosomes, this would lead to a reduction in CENP-A^{cnp1} nucleosomes at these regions (Figure S10). We also observed that the simultaneous overexpression of both CENP-A^{cnp1} and histone H3 results in a reduction in ectopic CENP-A^{cnp1}-GFP foci. Overexpression of H3 may reduce the pool of H4 available for assembly into CENP-A-H4 dimers, which results in decrease of CENP-A-containing nucleosomes available for noncentromeric sites (Figure S10). Furthermore, unlike H4 overexpression, which restores the growth rate of cells overexpressing CENP-A^{cnp1}, H3 overexpression aggravates this phenotype. This can be explained at least in part by a previous study showing that overexpressing H3 decreases CENP-A-H4 dimers at endogenous centromeres, leading to severe disruption of kinetochore and centromere activity (Castillo *et al.* 2007). Alternatively, the decrease in ectopic CENP-A^{cnp1} assembly caused by overexpression of H3 or H4 may result from nonspecific perturbations in chromatin assembly that interfere with the assembly of CENP-A^{cnp1} chromatin. Together, these data suggest that a delicate balance exists between the levels of CENP-A, H3, and H4, which is important for maintaining chromatin integrity at both centromeric and noncentromeric loci.

Acknowledgments

We thank Tokuko Haraguchi, Mitsuhiro Yanagida, and the Japan Yeast Genetic Resource Center for kindly providing the strains used in this study and W. Zacheus Cande, Jinpu Yang, and Xiaolan Zhao for critical reading of the manuscript. F.L. is a Pew Scholar in the Biomedical Sciences, supported by The Pew Charitable Trusts. The views expressed are those of the authors and do not necessarily reflect the views of The Pew Charitable Trusts. This project was supported by National Institutes of Health grant 1R01 GM106037 (to F.L.).

Literature Cited

Allshire, R. C., and G. H. Karpen, 2008 Epigenetic regulation of centromeric chromatin: Old dogs, new tricks? Nat. Rev. Genet. 9: 923–937.

Amato, A., T. Schillaci, L. Lentini, and A. Di Leonardo, 2009 CENPA overexpression promotes genome instability in pRb-depleted human cells. Mol. Cancer 8: 119.

Asakawa, H., T. Kojidani, C. Mori, H. Osakada, M. Sato *et al.*, 2010 Virtual breakdown of the nuclear envelope in fission yeast meiosis. Curr. Biol. 20: 1919–1925.

Au, W. C., A. R. Dawson, D. W. Rawson, S. B. Taylor, R. E. Baker *et al.*, 2013 A novel role of the N terminus of budding yeast histone H3 variant Cse4 in ubiquitin-mediated proteolysis. Genetics 194: 513–518.

Bassett, E. A., J. DeNizio, M. C. Barnhart-Dailey, T. Panchenko, N. Sekulic *et al.*, 2012 HJURP uses distinct CENP-A surfaces to recognize and to stabilize CENP-A/histone H4 for centromere assembly. Dev. Cell 22: 749–762.

Bernad, R., P. Sanchez, T. Rivera, M. Rodriguez-Corsino, E. Boyarchuk *et al.*, 2011 Xenopus HJURP and condensin II are required for CENP-A assembly. J. Cell Biol. 192: 569–582.

Black, B. E., and D. W. Cleveland, 2011 Epigenetic centromere propagation and the nature of CENP-a nucleosomes. Cell 144: 471–479.

Black, B. E., D. R. Foltz, S. Chakravarthy, K. Luger, V. L. Woods, Jr. *et al.*, 2004 Structural determinants for generating centromeric chromatin. Nature 430: 578–582.

Black, B. E., L. E. Jansen, P. S. Maddox, D. R. Foltz, A. B. Desai *et al.*, 2007 Centromere identity maintained by nucleosomes assembled with histone H3 containing the CENP-A targeting domain. Mol. Cell 25: 309–322.

Burrack, L. S., and J. Berman, 2012 Neocentromeres and epigenetically inherited features of centromeres. Chromosome Res. 20: 607–619.

Carroll, C. W., and A. F. Straight, 2006 Centromere formation: from epigenetics to self-assembly. Trends Cell Biol. 16: 70–78.

Carroll, C. W., M. C. Silva, K. M. Godek, L. E. Jansen, and A. F. Straight, 2009 Centromere assembly requires the direct recognition of CENP-A nucleosomes by CENP-N. Nat. Cell Biol. 11: 896–902.

Castillo, A. G., B. G. Mellone, J. F. Partridge, W. Richardson, G. L. Hamilton *et al.*, 2007 Plasticity of fission yeast CENP-A chromatin driven by relative levels of histone H3 and H4. PLoS Genet. 3: e121.

Castillo, A. G., A. L. Pidoux, S. Catania, M. Durand-Dubief, E. S. Choi *et al.*, 2013 Telomeric repeats facilitate CENP-A(Cnp1) incorporation via telomere binding proteins. PLoS ONE 8: e69673.

Chikashige, Y., D. Q. Ding, H. Funabiki, T. Haraguchi, S. Mashiko *et al.*, 1994 Telomere-led premeiotic chromosome movement in fission yeast. Science 264: 270–273.

Choi, E. S., A. Stralfors, S. Catania, A. G. Castillo, J. P. Svensson *et al.*, 2012 Factors that promote H3 chromatin integrity during transcription prevent promiscuous deposition of CENP-A (Cnp1) in fission yeast. PLoS Genet. 8: e1002985.

Clarke, L., and J. Carbon, 1980 Isolation of a yeast centromere and construction of functional small circular chromosomes. Nature 287: 504–509.

Collins, K. A., S. Furuyama, and S. Biggins, 2004 Proteolysis contributes to the exclusive centromere localization of the yeast Cse4/CENP-A histone H3 variant. Curr. Biol. 14: 1968–1972.

Cottarel, G., J. H. Shero, P. Hieter, and J. H. Hegemann, 1989 A 125-base-pair CEN6 DNA fragment is sufficient for complete meiotic and mitotic centromere functions in Saccharomyces cerevisiae. Mol. Cell. Biol. 9: 3342–3349.

Crotti, L. B., and M. A. Basrai, 2004 Functional roles for evolutionarily conserved Spt4p at centromeres and heterochromatin in Saccharomyces cerevisiae. EMBO J. 23: 1804–1814.

du Sart, D., M. R. Cancilla, E. Earle, J. I. Mao, R. Saffery *et al.*, 1997 A functional neo-centromere formed through activation of a latent human centromere and consisting of non-alpha-satellite DNA. Nat. Genet. 16: 144–153.

Fachinetti, D., H. D. Folco, Y. Nechemia-Arbely, L. P. Valente, K. Nguyen *et al.*, 2013 A two-step mechanism for epigenetic specification of centromere identity and function. Nat. Cell Biol. 15: 1056–1066.

Foltz, D. R., L. E. Jansen, A. O. Bailey, J. R. Yates, III, E. A. Bassett *et al.*, 2009 Centromere-specific assembly of CENP-a nucleosomes is mediated by HJURP. Cell 137: 472–484.

Fu, S., Z. Lv, Z. Gao, H. Wu, J. Pang *et al.*, 2013 De novo centromere formation on a chromosome fragment in maize. Proc. Natl. Acad. Sci. USA 110: 6033–6036.

Gonzalez, M., H. He, S. Sun, C. Li, and F. Li, 2013 Cell cycle-dependent deposition of CENP-A requires the Dos1/2-Cdc20 complex. Proc. Natl. Acad. Sci. USA 110: 606–611.

He, H., M. Gonzalez, F. Zhang, and F. Li, 2014 DNA replication components as regulators of epigenetic inheritance: lesson from fission yeast centromere. Protein Cell 5: 411–419.

Henikoff, S., and T. Furuyama, 2010 Epigenetic inheritance of centromeres. Cold Spring Harb. Symp. Quant. Biol. 75: 51–60.

Henikoff, S., and T. Furuyama, 2012 The unconventional structure of centromeric nucleosomes. Chromosoma 121: 341–352.

Henikoff, S., K. Ahmad, and H. S. Malik, 2001 The centromere paradox: stable inheritance with rapidly evolving DNA. Science 293: 1098–1102.

Heun, P., S. Erhardt, M. D. Blower, S. Weiss, A. D. Skora *et al.*, 2006 Mislocalization of the Drosophila centromere-specific histone CID promotes formation of functional ectopic kinetochores. Dev. Cell 10: 303–315.

Hewawasam, G., M. Shivaraju, M. Mattingly, S. Venkatesh, S. Martin-Brown *et al.*, 2010 Psh1 is an E3 ubiquitin ligase that targets the centromeric histone variant Cse4. Mol. Cell 40: 444–454.

Hiatt, E. N., E. K. Kentner, and R. K. Dawe, 2002 Independently regulated neocentromere activity of two classes of tandem repeat arrays. Plant Cell 14: 407–420.

Hiraoka, Y., T. Toda, and M. Yanagida, 1984 The NDA3 gene of fission yeast encodes beta-tubulin: a cold-sensitive nda3 mutation reversibly blocks spindle formation and chromosome movement in mitosis. Cell 39: 349–358.

Ishii, K., Y. Ogiyama, Y. Chikashige, S. Soejima, F. Masuda *et al.*, 2008 Heterochromatin integrity affects chromosome reorganization after centromere dysfunction. Science 321: 1088–1091.

Kagansky, A., H. D. Folco, R. Almeida, A. L. Pidoux, A. Boukaba *et al.*, 2009 Synthetic heterochromatin bypasses RNAi and centromeric repeats to establish functional centromeres. Science 324: 1716–1719.

Kearsey, S. E., S. Montgomery, K. Labib, and K. Lindner, 2000 Chromatin binding of the fission yeast replication factor mcm4 occurs during anaphase and requires ORC and cdc18. EMBO J. 19: 1681–1690.

Ketel, C., H. S. Wang, M. McClellan, K. Bouchonville, A. Selmecki *et al.*, 2009 Neocentromeres form efficiently at multiple possible loci in Candida albicans. PLoS Genet. 5: e1000400.

Krassovsky, K., J. G. Henikoff, and S. Henikoff, 2012 Tripartite organization of centromeric chromatin in budding yeast. Proc. Natl. Acad. Sci. USA 109: 243–248.

Lando, D., U. Endesfelder, H. Berger, L. Subramanian, P. D. Dunne *et al.*, 2012 Quantitative single-molecule microscopy reveals that CENP-A(Cnp1) deposition occurs during G2 in fission yeast. Open Biol 2: 120078.

Lefrancois, P., R. K. Auerbach, C. M. Yellman, G. S. Roeder, and M. Snyder, 2013 Centromere-like regions in the budding yeast genome. PLoS Genet. 9: e1003209.

Li, F., D. B. Goto, M. Zaratiegui, X. Tang, R. Martienssen *et al.*, 2005 Two novel proteins, Dos1 and Dos2, interact with Rik1 to regulate heterochromatic RNA interference and histone modification. Curr. Biol. 15: 1448–1457.

Li, F., M. Huarte, M. Zaratiegui, M. W. Vaughn, Y. Shi *et al.*, 2008 Lid2 is required for coordinating H3K4 and H3K9 methylation of heterochromatin and euchromatin. Cell 135: 272–283.

Li, Y. M., X. H. Liu, X. Z. Cao, L. Wang, and M. H. Zhu, 2007 [Expression of centromere protein A in hepatocellular carcinoma]. In Chinese. Zhonghua Bing Li Xue Za Zhi 36: 175–178.

Liu, X., I. McLeod, S. Anderson, J. R. Yates, III, and X. He, 2005 Molecular analysis of kinetochore architecture in fission yeast. EMBO J. 24: 2919–2930.

Maddox, P. S., K. D. Corbett, and A. Desai, 2012 Structure, assembly and reading of centromeric chromatin. Curr. Opin. Genet. Dev. 22: 139–147.

Maggert, K. A., and G. H. Karpen, 2001 The activation of a neocentromere in Drosophila requires proximity to an endogenous centromere. Genetics 158: 1615–1628.

Marshall, O. J., A. C. Chueh, L. H. Wong, and K. H. Choo, 2008 Neocentromeres: new insights into centromere structure,

disease development, and karyotype evolution. Am. J. Hum. Genet. 82: 261–282.
Maundrell, K., 1990 nmt1 of fission yeast. A highly transcribed gene completely repressed by thiamine. J. Biol. Chem. 265: 10857–10864.
Moreno, S., A. Klar, and P. Nurse, 1991 Molecular genetic analysis of fission yeast Schizosaccharomyces pombe. Methods Enzymol. 194: 795–823.
Moreno-Moreno, O., M. Torras-Llort, and F. Azorin, 2006 Proteolysis restricts localization of CID, the centromere-specific histone H3 variant of Drosophila, to centromeres. Nucleic Acids Res. 34: 6247–6255.
Muller, S., and G. Almouzni, 2014 A network of players in H3 histone variant deposition and maintenance at centromeres. Biochim. Biophys. Acta 1839: 241–250.
Murphy, T. D., and G. H. Karpen, 1995 Localization of centromere function in a Drosophila minichromosome. Cell 82: 599–609.
Nakayama, J., J. C. Rice, B. D. Strahl, C. D. Allis, and S. I. Grewal, 2001 Role of histone H3 lysine 9 methylation in epigenetic control of heterochromatin assembly. Science 292: 110–113.
Olszak, A. M., D. van Essen, A. J. Pereira, S. Diehl, T. Manke *et al.*, 2011 Heterochromatin boundaries are hotspots for de novo kinetochore formation. Nat. Cell Biol. 13: 799–808.
Palmer, D. K., K. O'Day, H. L. Trong, H. Charbonneau, and R. L. Margolis, 1991 Purification of the centromere-specific protein CENP-A and demonstration that it is a distinctive histone. Proc. Natl. Acad. Sci. USA 88: 3734–3738.
Pluta, A. F., A. M. Mackay, A. M. Ainsztein, I. G. Goldberg, and W. C. Earnshaw, 1995 The centromere: hub of chromosomal activities. Science 270: 1591–1594.
Ranjitkar, P., M. O. Press, X. Yi, R. Baker, M. J. MacCoss *et al.*, 2010 An E3 ubiquitin ligase prevents ectopic localization of the centromeric histone H3 variant via the centromere targeting domain. Mol. Cell 40: 455–464.
Sanchez-Perez, I., S. J. Renwick, K. Crawley, I. Karig, V. Buck *et al.*, 2005 The DASH complex and Klp5/Klp6 kinesin coordinate bipolar chromosome attachment in fission yeast. EMBO J. 24: 2931–2943.
Scott, K. C., and B. A. Sullivan, 2014 Neocentromeres: a place for everything and everything in its place. Trends Genet. 30: 66–74.
Sekulic, N., E. A. Bassett, D. J. Rogers, and B. E. Black, 2010 The structure of (CENP-A-H4)(2) reveals physical features that mark centromeres. Nature 467: 347–351.
Shang, W. H., T. Hori, N. M. Martins, A. Toyoda, S. Misu *et al.*, 2013 Chromosome engineering allows the efficient isolation of vertebrate neocentromeres. Dev. Cell 24: 635–648.
Smith, K. M., P. A. Phatale, C. M. Sullivan, K. R. Pomraning, and M. Freitag, 2011 Heterochromatin is required for normal distribution of Neurospora crassa CenH3. Mol. Cell. Biol. 31: 2528–2542.
Takayama, Y., H. Sato, S. Saitoh, Y. Ogiyama, F. Masuda *et al.*, 2008 Biphasic incorporation of centromeric histone CENP-A in fission yeast. Mol. Biol. Cell 19: 682–690.
Tomonaga, T., K. Matsushita, S. Yamaguchi, T. Oohashi, H. Shimada *et al.*, 2003 Overexpression and mistargeting of centromere protein-A in human primary colorectal cancer. Cancer Res. 63: 3511–3516.
Topp, C. N., R. J. Okagaki, J. R. Melo, R. G. Kynast, R. L. Phillips *et al.*, 2009 Identification of a maize neocentromere in an oat-maize addition line. Cytogenet. Genome Res. 124: 228–238.
Warburton, P. E., 2004 Chromosomal dynamics of human neocentromere formation. Chromosome Res. 12: 617–626.
Westermann, S., and A. Schleiffer, 2013 Family matters: structural and functional conservation of centromere-associated proteins from yeast to humans. Trends Cell Biol. 23: 260–269.
Zhang, W., B. Friebe, B. S. Gill, and J. Jiang, 2010 Centromere inactivation and epigenetic modifications of a plant chromosome with three functional centromeres. Chromosoma 119: 553–563.

Communicating editor: S. E. Bickel

GENETICS

Supporting Information
http://www.genetics.org/lookup/suppl/doi:10.1534/genetics.114.171173/-/DC1

Ectopic Centromere Nucleation by CENP-A in Fission Yeast

Marlyn Gonzalez, Haijin He, Qianhua Dong, Siyu Sun, and Fei Li

Copyright © 2014 by the Genetics Society of America
DOI: 10.1534/genetics.114.171173

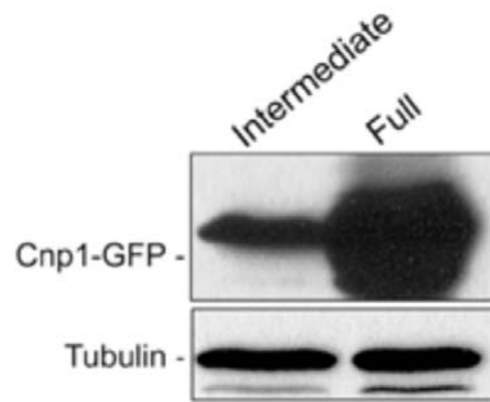

Figure S1 Western blot analysis of diluted cell extracts from intermediately-induced or fully-induced cells carrying *nmt1*-CENP-A^{cnp1}-GFP. Whole-cell extracts were subjected to immunoblotting with anti-GFP antibody. Tubulin was used as a loading control.

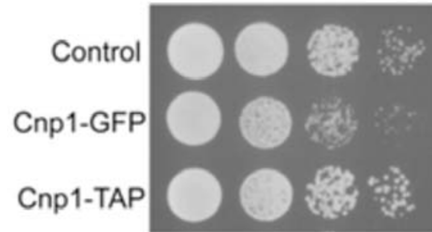

Figure S2 Replacing endogenous CENP-A^{cnp1} with CENP-A^{cnp1}-GFP only causes a slightly slower growth rate than wild-type cells, while replacing endogenous CENP-A^{cnp1} with CENP-A^{cnp1}-TAP results in no obvious growth defect. These results indicate that both CENP-A^{cnp1}-GFP and CENP-A^{cnp1}-TAP are largely functional. Serial dilutions of indicated strains were plated in rich YES medium.

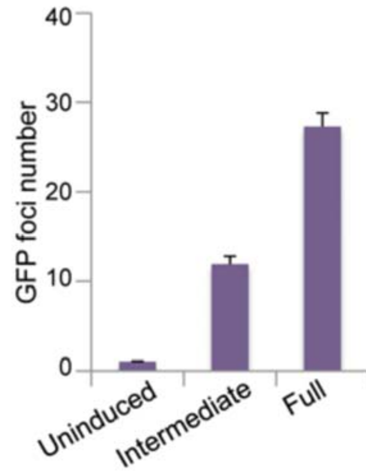

Figure S3 The average number of GFP foci in uninduced, intermediate, or fully-induced cells carrying *nmt1*-CENP-A^{cnp1}–GFP. Error bars indicate standard deviation (SD). Chromosome segregation defects in cells overexpressing CENP-A^{cnp1}-GFP were visualized by CENP-A^{cnp1} -GFP signal.

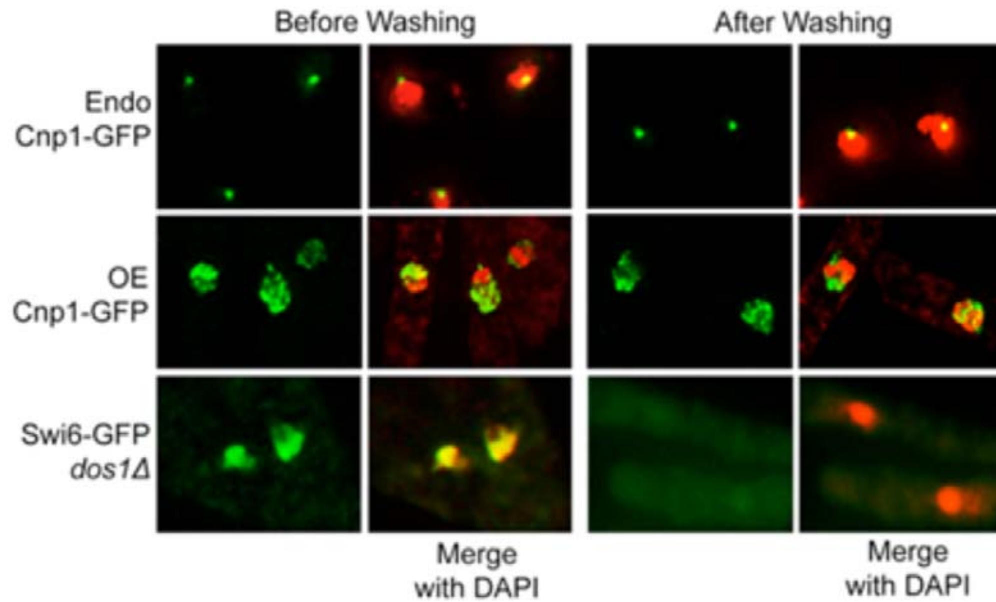

Figure S4 *in situ* chromatin-binding assay for overexpressed CENP-A^{cnp1}-GFP. Wild type cells expressing endogenous CENP-A^{cnp1}-GFP and the *dos1*Δ mutant expressing Swi6-GFP were used as a positive and negative control, respectively. After washing with Triton X-100, both endogenous (top panel) and ectopic CENP-A^{cnp1}-GFP foci (middle panel) remained in the nucleus, indicating that ectopically localized CENP-A^{cnp1}-GFP associates with chromatin. In contrast, the Swi6-GFP signal in the *dos1*Δ mutant was completely washed away from nucleus (lower panel) as expected.

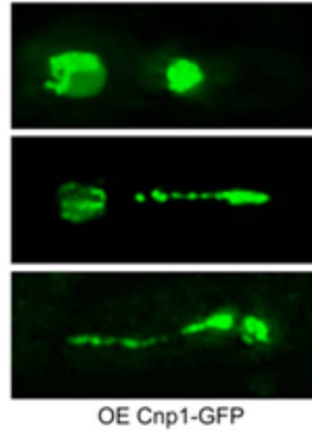

Figure S5 Chromosome segregation defects in cells overexpressing CENP-A^{cnp1}-GFP were visualized by CENP-A^{cnp1} -GFP signal.

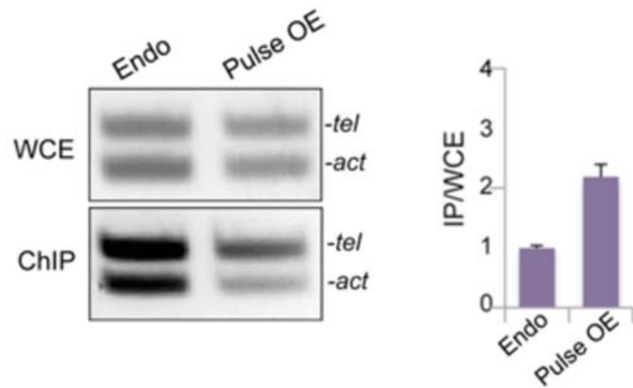

Figure S6 ChIP assays show that CENP-A^{cnp1}-GFP is still enriched at heterochromatin boundaries 24 hours after pulse overexpression of CENP-A^{cnp1}-GFP. ChIP assays were performed using an antibody against GFP. Immunoprecipitated DNA was analyzed by competitive PCR with primers specific for a sub-telomeric region (*tel*) and a control gene, *act1$^+$* (*act*). WCE, whole-cell extracts.

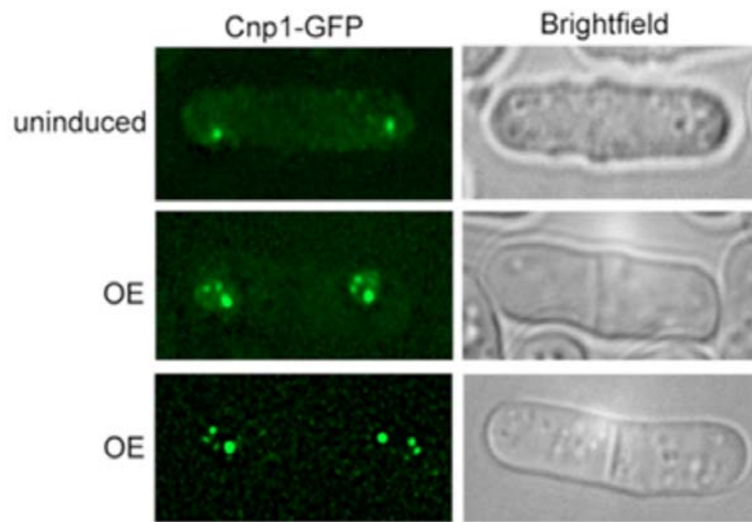

Figure S7 Daughter nuclei from pulse-induced cells carrying CENP-A^{cnp1}–GFP show multiple CENP-A^{cnp1}–GFP foci 18 hrs after addition of thiamine. In contrast, daughter nuclei from cells expressing endogenous-level CENP-A^{cnp1}–GFP display single CENP-A^{cnp1}–GFP focus (top panel). Note that daughter nuclei from a pulsed-induced cell (middle and bottom panels) show near identical distribution of ectopic CENP-A-GFP foci, suggesting inheritance of these sites from mother to daughter cells.

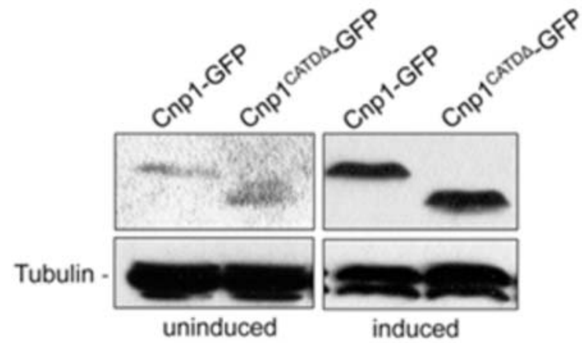

Figure S8 Western blot analysis of total cell extracts from cells carrying *nmt1*-CENP-A^{cnp1}-GFP or *nmt1*-Cnp1^{CATDΔ}-GFP at either uninduced or induced condition. Note that the cell extracts from induced cells were diluted in order to compare protein abundances. Whole-cell extracts were subjected to immunoblotting with anti-GFP antibody. Tubulin was used as a loading control.

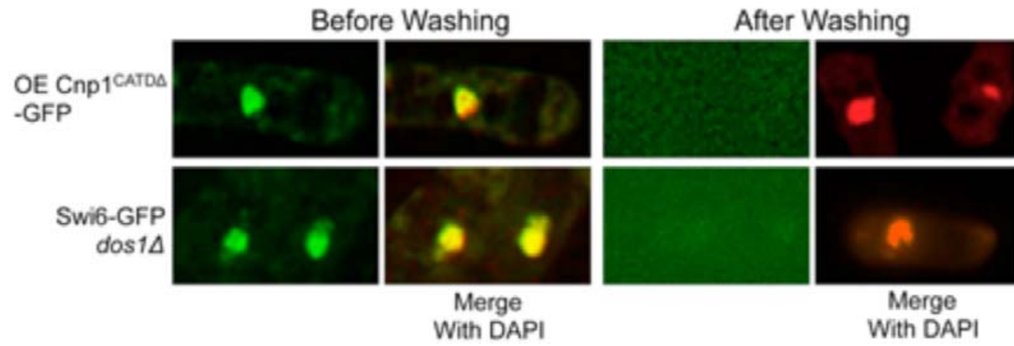

Figure S9 *in situ* chromatin-binding assay for wild type cells overexpressing Cnp1^{CATDΔ}-GFP. After washing with Triton X-100, the Cnp1^{CATDΔ}-GFP signal was washed away from nucleus, indicating that the Cnp1^{CATDΔ}-GFP does not associate with chromatin (top panel). The *dos1*Δ mutant expressing GFP-Swi6 were used as a control (lower panel).

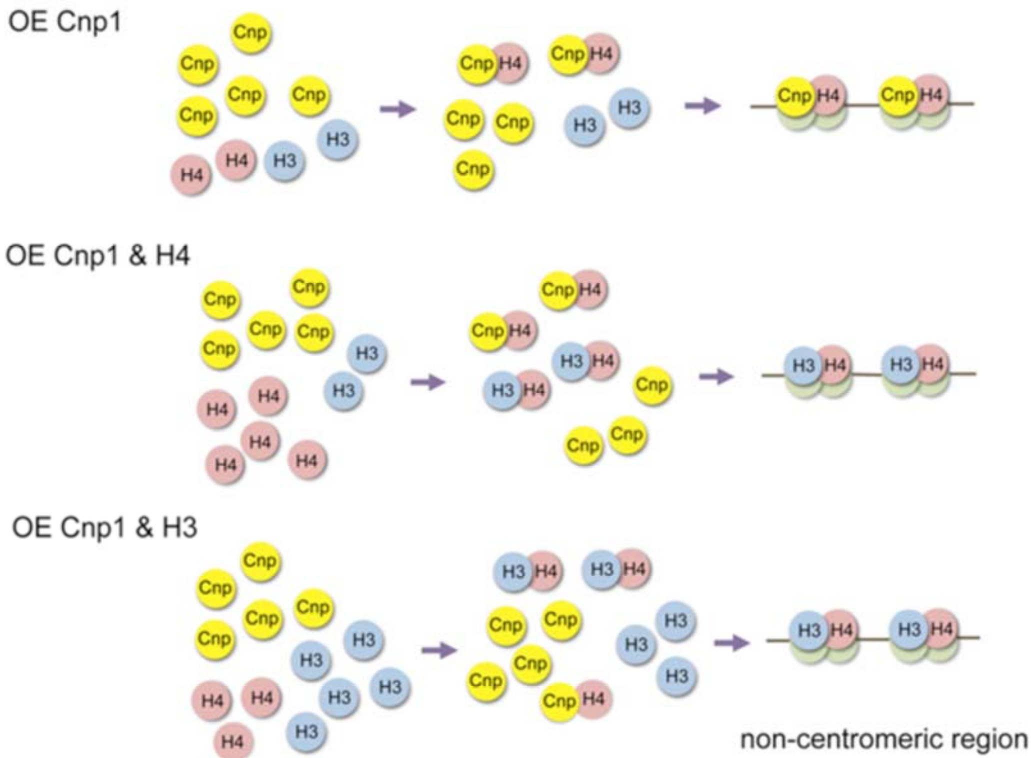

Figure S10 Model: Overexpression of histone H3 and H4 disrupts the assembly of CENP-A at ectopic sites. In cells overexpressing CENP-A^{cnp1} alone, excess CENP-A^{cnp1} outcompetes histone H3 for H4, leading to overabundant CENP-A-H4 dimers and insufficient H3-H4 dimers. Consequently, CENP-A^{cnp1} nucleosomes are assembled at non-centromeric regions. In cells overexpressing both H4 and CENP-A^{cnp1}, sufficient H4 is available to increase the pool of H3-H4 dimers. Since non-centromeric chromatin preferentially assembles H3 over CENP-A^{cnp1}-containing nucleosomes, H3 rather than CENP-A^{cnp1} is deposited to non-centromeric region. In cells overexpressing both H3 and CENP-A^{cnp1}, excess H3 effectively outcompetes CENP-A^{cnp1} for H4. This results in less CENP-A^{cnp1}-nucleosomes forming, and the consequent depletion of CENP-A^{cnp1} at non-centromeric sites, as well as at centromeres.

Table S1 Strains used in this study.

Strain	Genotype
FL29	*h^{90} Δdos1::ura4 ars1(Mlul)::pREP81X-GFP-Swi6 leu1-32 ura4-D18*
FL554	*h^{-} pREP1-CENP-A^{cnp1}-GFP ura4D-18 leu1-32 ade6-210 his3-D1*
FL555	*h^{-} pREP1-$Cnp1^{N\Delta}$-GFP ura4D-18 leu1-32 ade6-210 his3-D1*
FL556	*h^{-} pREP1-$Cnp1^{CATD\Delta}$-GFP ura4D-18 leu1-32 ade6-210 his3-D1*
FL557	*h^{-} pREP2-dos1-GFP pREP1 ura4D-18 leu1-32 ade6-210 his3-D1*
FL558	*h^{-} pREP1-CENP-A^{cnp1}-GFP pREP2 ura4D-18 leu1-32 ade6-210 his3-D1*
FL560	*h^{-} Δdos1::KanMX pREP1 pREP2 ura4D-18 leu1-32 ade6-210 his3-D1*
FL561	*h^{-} α-tub-GFP ish1-mCherry-hph ura4D-18 pREP2-CENP-A^{cnp1}-TAP*
FL562	*h^{-} CENP-A^{cnp1}-GFP::lys^{+} pREP1-CENP-A^{cnp1}-mCherry ura4D-18 leu1-32*
FL563	*h^{90} dad1::dad1-GFP-HA-kanMX pREP1-CENP-A^{cnp1}-mCherry ade6-216 leu1-32 lys1-131 ura4D-18*
FL564	*h^{90} ndc80::ndc80-GFP-HA-kanMX pREP1-CENP-A^{cnp1}-mCherry ade6-216 leu1-32 lys1-131 ura4D-18*
FL565	*h^{-} pREP2-H3 pREP1 ura4D-18 leu1-32 ade6-210 his3-D1*
FL566	*h^{-} pREP1-CENP-A^{cnp1}-GFP pREP2-H3 ura4D-18 leu1-32 ade6-210 his3-D1*
FL567	*h^{-} pREP2-H4 pREP1 ura4D-18 leu1-32 ade6-210 his3-D1*
FL568	*h^{-} pREP1-CENP-A^{cnp1}-GFP pREP2-H4 ura4D-18 leu1-32 ade6-210 his3-D1*
FL574	*h^{-} a-tub-GFP::lys pREP1-CENP-A^{cnp1}-mCherry leu1-32 ura4D-18*

Table S2 Primers used in this study.

Primer Name	Sequence
Act_1	ATGGAAGAAGAAATCGCAGCG
Act_2	ATGCCAAATCTTTTCCATATC
Tel1_R	ATAGCGGATCGTTTTTTAACGA
Rev_Tel1_R	GAAAAGCTGTTTATACGAACCTTAAT