

Induced Ectopic Kinetochores Bypasses the Requirement for CENP-A Nucleosomes

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SUMMARY

Accurate chromosome segregation requires assembly of the multiprotein kinetochore complex at centromeres. Although prior work identified the centromeric histone H3-variant CENP-A as the important upstream factor necessary for centromere specification, in human cells CENP-A is not sufficient for kinetochore assembly. Here, we demonstrate that two constitutive DNA-binding kinetochore components, CENP-C and CENP-T, function to direct kinetochore formation. Replacing the DNA-binding regions of CENP-C and CENP-T with alternate chromosome-targeting domains recruits these proteins to ectopic loci, resulting in CENP-A-independent kinetochore assembly. These ectopic kinetochore-like foci are functional based on the stoichiometric assembly of multiple kinetochore components, including the microtubule-binding KMN network, the presence of microtubule attachments, the microtubule-sensitive recruitment of the spindle checkpoint protein Mad2, and the segregation behavior of foci-containing chromosomes. We additionally find that CENP-T phosphorylation regulates the mitotic assembly of both endogenous and ectopic kinetochores. Thus, CENP-C and CENP-T form a critical regulated platform for vertebrate kinetochore assembly.

INTRODUCTION

During mitosis, cells must accurately partition their genome such that genetic information is transferred unperturbed to the progeny. In eukaryotes, accurate chromosome segregation requires each chromosome to interact appropriately with microtubules from the mitotic spindle. This interaction is mediated by the macromolecular kinetochore complex that forms upon centromere DNA (Cheeseman and Desai, 2008). Ensuring proper chromosome segregation and kinetochore function requires

three key activities. First, the centromere must be specified at a single site on each sister chromatid. Failure to specify a centromere prevents chromosome segregation, whereas multiple functional centromeric sites could result in chromosome fragmentation due to inappropriate attachments to the spindle. Second, a specific chromosome structure surrounding the centromere must be generated to ensure that this region can resist spindle forces and prevent chromosomal twisting that would result in merotelically attached kinetochores. Third, the macromolecular kinetochore structure, composed of almost 100 different proteins (Cheeseman and Desai, 2008), must assemble upon the centromere in a cell cycle-regulated manner to generate a structure capable of binding to microtubules. These proteins include the KNL1/Mis12 complex/Ndc80 complex (KMN) network, which is a core component of the outer kinetochore-microtubule interface (Cheeseman and Desai, 2008).

Previous work defined the histone H3 variant CENP-A (also called CenH3), which forms specialized nucleosomes found predominantly at centromeres, as the key upstream factor required for kinetochore specification (Gascoigne and Cheeseman, 2011). However, the mechanisms that act to direct the assembly of the remaining kinetochore proteins remain largely unknown (Gascoigne and Cheeseman, 2011). Despite the requirement for CENP-A in specifying the site of kinetochore assembly (Howman et al., 2000), it is unclear whether CENP-A acts directly in kinetochore assembly as a platform to associate with other kinetochore proteins (Carroll et al., 2009, 2010) or if it generates a specialized chromatin environment that is permissive for kinetochore formation. In *Drosophila*, CENP-A is sufficient to drive kinetochore assembly because its ectopic localization causes the formation of additional kinetochore structures (Heun et al., 2006). In contrast, in human cells the presence of CENP-A does not appear to be sufficient for kinetochore assembly (Van Hooser et al., 2001).

In addition to CENP-A, a group of 16 chromatin-proximal proteins termed the constitutive centromere-associated network (CCAN) is present at centromeres in vertebrate cells throughout the cell cycle (Amano et al., 2009; Foltz et al., 2006; Hori et al., 2008; Okada et al., 2006; reviewed in Cheeseman and Desai, 2008) and is required for correct formation of the mitotic kinetochore. However, the precise role of the CCAN in kinetochore

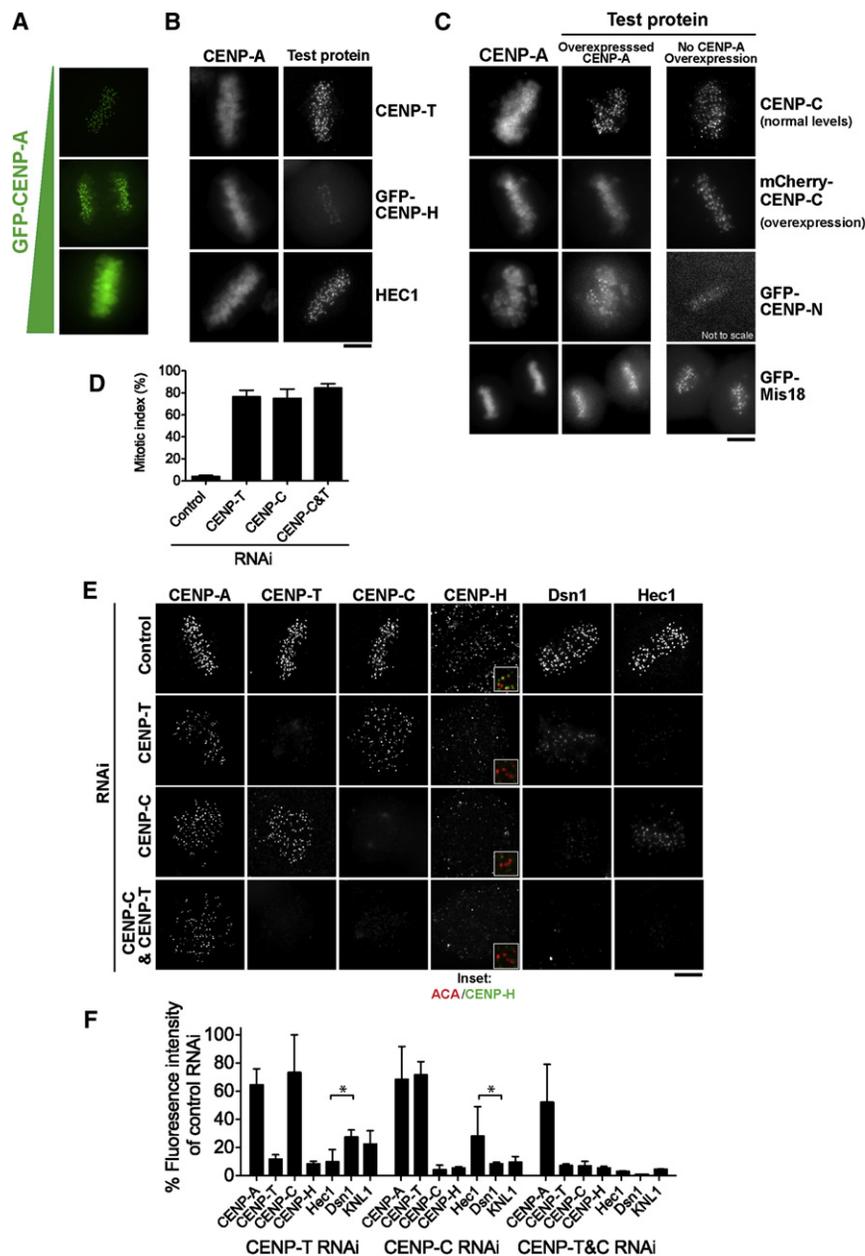


Figure 1. Differential Roles of CENP-A, the CENP-T/W Complex, and CENP-C in Kinetochores Assembly

(A) Images of HeLa cells with increasing levels of GFP-CENP-A expression. CENP-A localizes to chromosome arms at high expression levels.

(B) Representative images of kinetochores components *not* mislocalized by CENP-A overexpression, visualized using anti-CENP-T and anti-Hec1 antibodies, or GFP^{LAP}-CENP-H.

(C) Images showing mislocalization of kinetochores components to chromosome arms in the presence of ectopic CENP-A relative to a GFP-H2B control. CENP-C was visualized by immunofluorescence, or by co-overexpression of mCherry-CENP-C. CENP-N or Mis18 was visualized using HeLa cell lines stably expressing GFP^{LAP} fusions. (D) Graph showing quantification of mitotic index 48 hr after the indicated depletions by RNAi ($n = 100$ cells/condition, \pm standard error of the mean [SEM]).

(E) Representative immunofluorescence images of HeLa cells 48 hr after RNAi depletion of the indicated proteins. Merge insets show ACA (red) and CENP-H (green).

(F) Quantification of kinetochore intensity 48 hr following RNAi depletion of CENP-C, CENP-T, or CENP-C and CENP-T ($n > 50$ kinetochores, ≥ 5 cells, \pm standard deviation [SD]). Asterisk indicates significant difference as determined by Mann Whitney U test ($p < 0.005$).

Scale bars, 5 μ m. Also see Figure S1.

targeting of CENP-C and CENP-T can generate foci that function as kinetochores-like structures in the absence of CENP-A, demonstrating that CENP-C and the CENP-T/W complex function as core kinetochores assembly factors in vertebrate cells.

RESULTS

CENP-A Mistargeting Results in the Ectopic Recruitment of a Small Subset of Kinetochores Proteins

Specialized centromeric CENP-A nucleosomes are thought to provide the foundation

for kinetochores assembly. To test the sufficiency of CENP-A for directing kinetochores assembly, we induced the misincorporation of GFP-CENP-A into noncentromeric chromatin by overexpression in HeLa cells (Figure 1A). Ectopically incorporated CENP-A resulted in the corecruitment of only three proteins (CENP-C, CENP-N, and Mis18; Figure 1C) out of 16 kinetochores proteins tested (Figure 1B; see Figure S1 available online). Thus, in human cells, mistargeting of CENP-A does not result in ectopic kinetochores formation. CENP-C and CENP-N bind to CENP-A *in vitro* (Carroll et al., 2009, 2010), and Mis18 functions as a CENP-A licensing factor (Fujita et al., 2007; Hayashi et al., 2004). In contrast to previous observations (Van Hooser et al., 2001), mistargeting of CENP-C required co-overexpression

assembly remains unclear. Several CCAN proteins, including the CENP-T/W complex and CENP-C, have been shown to possess DNA-binding activity (Hori et al., 2008; Sugimoto et al., 1994), whereas others such as CENP-N interact directly with CENP-A containing nucleosomes (Carroll et al., 2009, 2010). Defining how these proteins form the interface between centromeric chromatin and the outer kinetochores microtubule-binding proteins and how kinetochores assembly is regulated to recruit outer kinetochores components during mitosis is a key goal.

To define the mechanisms that direct kinetochores assembly downstream of CENP-A, we analyzed the constitutive kinetochores components, focusing on the DNA-binding proteins CENP-C and the CENP-T/W complex. We find that ectopic

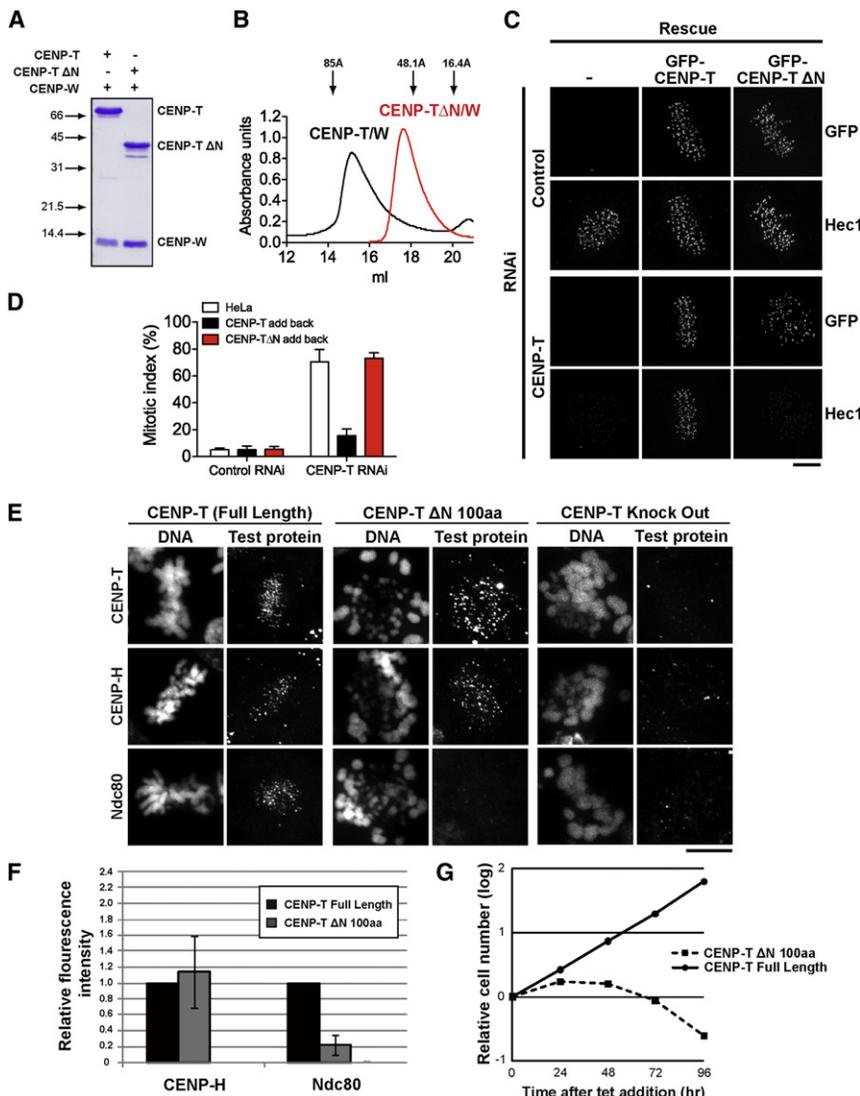


Figure 2. The CENP-T N Terminus Is Required for Kinetochores Assembly

(A) Coomassie-stained gel showing bacterially purified recombinant full-length 6xHis-CENP-T/CENP-W or CENP-T-ΔN/CENP-W, lacking aa 1–288.

(B) Graph showing the elution profile (optical density [OD]₂₈₀) of recombinant CENP-T/W complex and CENP-T-ΔN/W on a Superose 6 size-exclusion column. Arrows indicate the migration of standards with known Stokes radii: thyroglobulin (85 Å), aldolase (48.1 Å), and RNase A (16.4 Å).

(C) Immunofluorescence images showing levels of Hec1/Ndc80 48 hr after RNAi depletion of CENP-T in HeLa cells or HeLa cell lines expressing RNAi-resistant GFP-CENP-T or GFP-CENP-T-ΔN. Scale bar, 5 μm.

(D) Graph showing quantification of the mitotic index 48 hr after RNAi depletion of CENP-T in HeLa cells, or HeLa cell lines expressing RNAi-resistant CENP-T or CENP-T ΔN (n = 100 cells, ±SEM).

(E) Representative immunofluorescence images of chicken DT40 cells after depletion of endogenous CENP-T and expression of the indicated protein fragments. Scale bar, 10 μm.

(F) Quantification of kinetochore intensity after depletion of CENP-T and expression of the indicated proteins (n = 200, ±SD).

(G) Graph showing viability of CENP-T conditional depletion in DT40 cells expressing the indicated proteins after addition of tetracycline.

Also see Figure S2.

of mCherry-CENP-C and GFP-CENP-A. CENP-A/CENP-C co-overexpression resulted in the mistargeting of some additional kinetochore proteins, such as the Mis12 complex subunit Dsn1, to chromosome arms (Figure S1B), but this occurred with lower frequency and at low levels. Thus, although CENP-A is essential for centromere specification, these data indicate that it is not sufficient for kinetochore assembly in humans.

CENP-T/W and CENP-C Are Essential for Kinetochores Assembly

We next sought to define the contributions of other chromatin proximal proteins to kinetochore assembly. Depletion of CENP-C or CENP-T individually or in combination led to a mitotic arrest but caused only a mild reduction in CENP-A and the reciprocal protein at centromeres (Figures 1D–1F), suggesting that these essential DNA-binding proteins are recruited independently. Importantly, codepletion of CENP-C and CENP-T compromised the localization of all other kinetochore proteins

differences in their relative requirement for the recruitment of different KMN network components (Figure 1F). Thus, CENP-C and CENP-T are both required for kinetochore assembly.

The CENP-T N Terminus Is Required for Kinetochores Assembly

The DNA-binding activity of CENP-T resides in a histone-like fold at its C terminus (Hori et al., 2008; our unpublished results). Size-exclusion chromatography of recombinant CENP-T/W complex indicated that it has an elongated structure, with a Stokes radius of ~5.5 nm (Figure 2B). Sucrose gradients indicated that the complex does not exist as an oligomer (Figure S2A), and removal of the N-terminal 288 amino acids dramatically shifted the elution profile of the complex (Figures 2A and 2B), suggesting that the N terminus of CENP-T has an extended structure. Analysis of the N terminus of CENP-T in vivo indicated that it is essential for kinetochore assembly and mitotic progression in both human cells and chicken DT40 cells. GFP-CENP-T ΔN terminus (lacking

amino acids 1–287 in human cells, or 1–100 in chicken DT40 cells) failed to rescue the mitotic arrest and delocalization of Hec1/Ndc80 from kinetochores following CENP-T depletion (Figures 2C–2G), suggesting that the N terminus of CENP-T functions in recruiting downstream kinetochore components.

Targeting of CENP-C and CENP-T to Ectopic Foci Is Sufficient for Core Kinetochore Assembly

To determine whether CENP-C or CENP-T/W activity is sufficient for aspects of kinetochore assembly, we developed a strategy to target these proteins to ectopic loci. Overexpression of CENP-T was not sufficient to cause ectopic localization (Figure S2B). Therefore, we replaced the C-terminal DNA-binding regions of CENP-T and CENP-C with alternate DNA-binding domains, allowing us to target these proteins either throughout the chromosome using fusions to histone H2B (Figure S3) or to a specific locus using a LacI fusion in a U2OS cell line with an array of Lac operator (LacO) repeats integrated into chromosome 1 (Janicki et al., 2004) (Figures 3A and 3B). In the experiments described below, the large number of LacO repeats present in this cell line resulted in foci that were 5- to 50-fold larger than endogenous kinetochores based on fluorescence intensity. Thus, whereas these ectopic LacI-directed foci allowed us to analyze the activity of CENP-T and CENP-C, these large foci are likely to differ from endogenous kinetochores.

In the presence of either GFP-CENP-C- Δ C or GFP-CENP-T- Δ C fusions, endogenous CENP-A, CENP-T, and CENP-C did not colocalize with the fusion proteins (Figure 3C; Figure S3A). In contrast, representative KMN network subunits Dsn1 (Mis12 complex), KNL1, and Ndc80/Hec1 colocalized with GFP-CENP-C- Δ C and GFP-CENP-T- Δ C fusions, but not with GFP controls (Figure 3D; Figure S3B). We also observed colocalization of Ndc80 and Nuf2 with GFP-CENP-T- Δ C-LacI fusions in chicken DT40 cells (Figure S3F). Importantly, simultaneous expression of mCherry-CENP-T- Δ C and GFP-CENP-C- Δ C fusions resulted in levels of KMN network components at ectopic foci that were stoichiometrically proportional to the amount of CENP-T and CENP-C present at the foci relative to endogenous kinetochores (Figures 3D and 3E; Figure S3B). For example if five times more CENP-T was present at an ectopic focus relative to an endogenous kinetochore, five times more Dsn1, KNL1, and Ndc80/Hec1 was also present. Thus, in the absence of CENP-A, CENP-T and CENP-C can properly assemble the microtubule-binding KMN network at noncentromeric sites.

As for our depletion analysis (Figure 1), we observed an asymmetric behavior in the mistargeting of KMN network components in this ectopic assay; CENP-C- Δ C fusions preferentially recruited Dsn1 and KNL1, whereas CENP-T- Δ C-fusions preferentially recruited Ndc80/Hec1 (Figures 3D and 3E; Figure S3B). Consistent with this, our biochemical analysis indicated that CENP-T/W can bind directly to a shortened Ndc80 complex (Ndc80^{Bonsai}; Ciferri et al., 2008), CENP-K, and weakly to the Mis12 complex (Figure 4A). Similarly, we found that CENP-C can interact with the Mis12 complex in vivo and in vitro (Figures 4B and 4C) (also see Przewłoka et al., 2011; Screpanti et al., 2011). These data demonstrate that the N-terminal regions of CENP-T and CENP-C can each act directly to recruit the KMN network to kinetochores during mitosis.

Induced Kinetochore-like Foci Recruit Multiple Kinetochore Components

Induced targeting of CENP-C and CENP-T to ectopic foci results in formation of kinetochore-like structures based on the recruitment of KMN network components (Figure 3). To evaluate the functionality of these ectopic kinetochore protein foci, we next tested for the presence of outer kinetochore and regulatory proteins (see Cheeseman and Desai, 2008). Multiple kinetochore proteins colocalized with GFP-CENP-C- Δ C-LacI and GFP-CENP-T- Δ C-LacI foci, but not GFP-LacI foci, including the KMN network-binding protein Zwint, the microtubule-binding Ska1 complex subunit Ska3, the kinetochore nucleoporin Nup107-160 complex subunit Nup133, and CENP-N (Figure 5A; Figure S4A). The majority of these proteins were observed at 100% of foci (Figure 5B), and Ska3 targeted to ectopic foci with a stoichiometry similar to that at endogenous kinetochores. We also obtained similar results using H2B fusions (Figure S3C).

We next examined CENP-T/C foci for the presence of kinetochore regulatory proteins. We found that Aurora B kinase and INCENP, components of the chromosomal passenger complex (CPC), localized to both ectopic foci (Figure 5A) and chromosome arms in the H2B fusions (Figure S3C). However, we note that Aurora B signals were only observed at 34% of mitotic LacO foci (Figure 5B). Consistent with the recruitment of Aurora B, Dsn1 present at ectopic LacO foci was phosphorylated at an established Aurora B phosphorylation site based on phosphoantibody localization (Figure 5C) (Welburn et al., 2010). Finally, we observed the MEI-S332/Shugoshin-family protein Sgo1 at ectopic CENP-T/C foci in 31% of cells. In contrast, several kinetochore proteins were not observed at ectopic CENP-T/C foci in U2OS LacO-containing cells, or H2B fusion protein-expressing cells, including CENP-O, MCAK, Bub1, and Mis18 (Figures S4B–S4F).

We also analyzed the ectopic kinetochore-like foci for components of the spindle assembly checkpoint (SAC), and observed the recruitment of Mad2 and ZW10 to ectopic CENP-T/C foci in human cells (Figures 5A and 5D), and Mad2, BubR1, CENP-E, and ZW10 in chicken DT40 cells (Figure 5E; data not shown). Importantly, we observed a dramatic increase in the recruitment of Mad2 to the ectopic CENP-T/C foci in absence of microtubules (Figure 5D), similar to what is observed at endogenous kinetochores. The targeting of these diverse regulatory kinetochore proteins, and the microtubule-sensitive recruitment of Mad2, is consistent with functional kinetochore-like structures at these sites.

Induced Kinetochore-like Foci Associate with Microtubules

We next tested the interaction of ectopic kinetochore-like foci with microtubules. Although GFP-LacI foci remain circular during mitosis, GFP-CENP-T- Δ C-LacI foci formed a bar-like shape, suggestive of a force being applied across the region (Figure 6A, arrow). Deformation of the GFP-CENP-T- Δ C-LacI focus was exacerbated by treatment with the Aurora B inhibitor ZM447439, which has been proposed to regulate both kinetochore-microtubule attachments (Cheeseman and Desai, 2008) and chromatin structure (Lipp et al., 2007). The deformed shape of the GFP-CENP-T- Δ C-LacI focus was dependent on

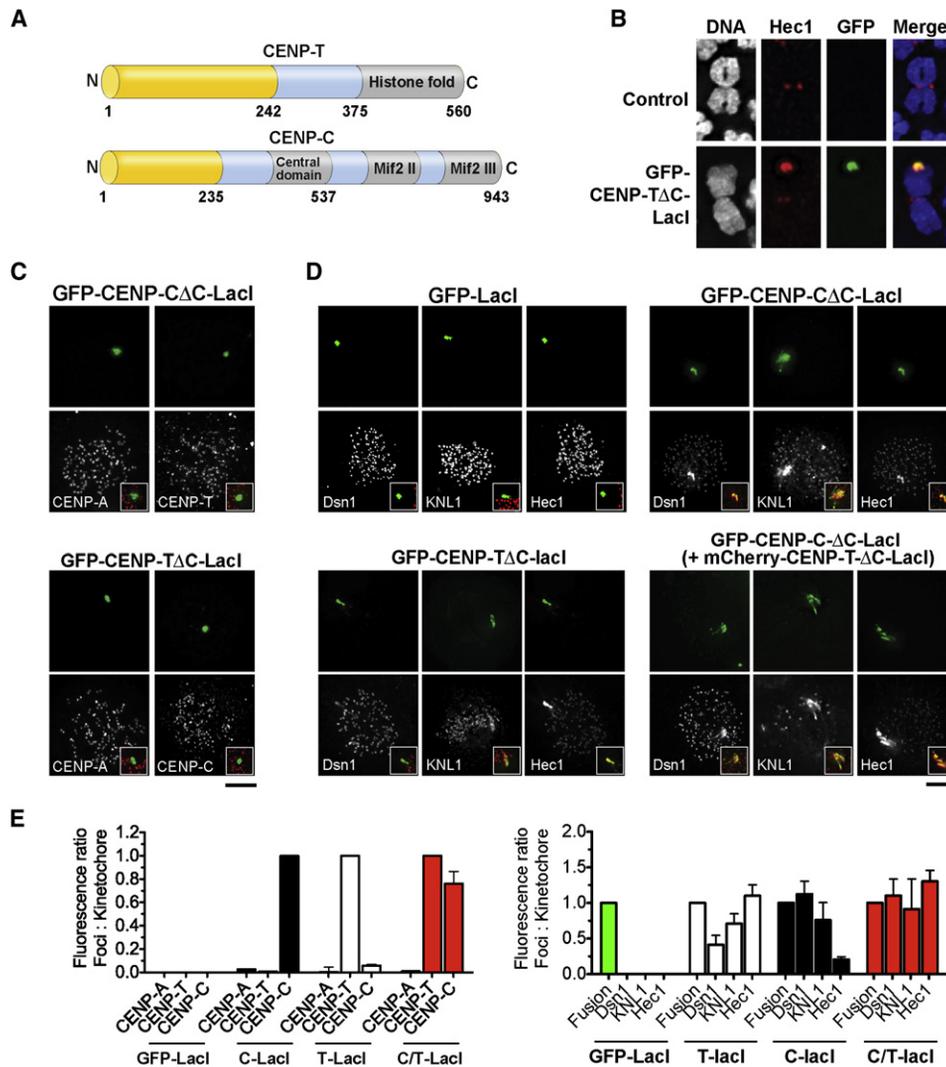


Figure 3. Targeting of CENP-C-LacI and CENP-T-LacI to an Ectopic Focus Causes Mislocalization of the KMN Network

(A) Schematic representation of CENP-T and CENP-C. The N-terminal regions used in fusion protein experiments are indicated in yellow (amino acids 1–242 for CENP-T and 1–235 for CENP-C). DNA-binding regions are indicated in gray (Hori et al., 2008; Yang et al., 1996).

(B) Immunofluorescence images showing representative metaphase chromosome spreads from U2OS-LacO cells. In the bottom panels chromosome 1 with GFP-CENP-T-ΔC-LacI present at the LacO array.

(C) Immunofluorescence images showing only centromeric localization of DNA-binding kinetochore proteins in cells transiently expressing GFP-CENP-T-ΔC-LacI or GFP-CENP-C-ΔC-LacI after 15 hr nocodazole treatment. Insets show a merge of kinetochore protein (red) and LacI foci (green).

(D) Immunofluorescence images showing colocalization of KMN network proteins with LacI foci in cells transiently expressing GFP-CENP-T-ΔC-LacI or GFP-CENP-C-ΔC-LacI, or coexpressing mCherry-CENP-T-ΔC-LacI (not shown) and GFP-CENP-C-ΔC-LacI. Insets show a merge of kinetochore protein (red) and LacI foci (green).

(E) Quantitation of the relative fluorescence of the indicated kinetochore proteins at endogenous kinetochores versus ectopic foci in cells expressing LacI fusion proteins. Quantification was conducted after 15 hr nocodazole treatments (>10 cells/condition, 20 kinetochores/cell, ±SEM). Data are shown normalized to the foci/kinetochore ratio of the fusion protein.

Also see Figure S3 and Movie S4.

microtubules because nocodazole treatment relaxed this to a circular morphology (Figure 6A). In the absence of endogenous kinetochores (using RNAi-based depletion of endogenous CENP-T and CENP-C), robust interactions with microtubules were observed for ectopic CENP-T/C-LacI foci, but not GFP-LacI controls (Figure 6A). These foci often appeared broken, indicating that interactions with microtubules are maintained and

may cause damage due to merotelic attachments. Electron microscopy analysis of CENP-T-LacI foci also indicated the presence of microtubule attachments at these sites (Figure 6B) and the formation of constriction similar to that found at endogenous kinetochores (Figure S5A). The interaction of CENP-T-based structures with microtubules does not appear to require the presence of chromosomal DNA, as when CENP-T was

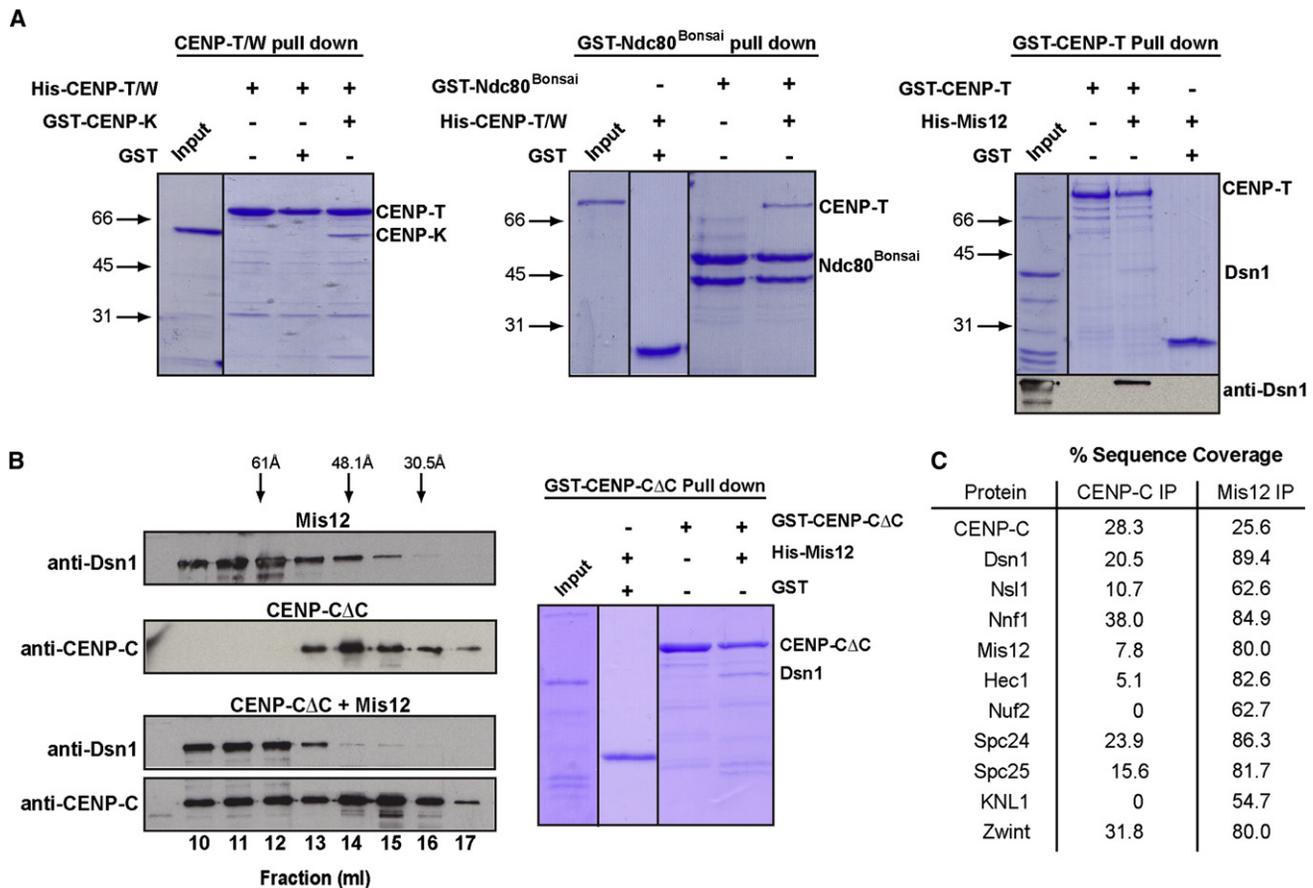


Figure 4. The CENP-T/W Complex and CENP-C Interact Directly with KMN Network Components In Vitro

(A) The CENP-T/W complex binds directly to CENP-K, GST-Ndc80^{Bonsai}, and the Mis12 complex in vitro. In the left panel is a Coomassie-stained gel showing 6xHis-CENP-T/W complex immobilized on Ni-NTA agarose resin, alone or in the presence of GST or GST-CENP-K. In the middle panel is a Coomassie-stained gel showing 6xHis-CENP-T/W complex binding to either GST-Ndc80^{Bonsai} or GST immobilized on glutathione agarose. In the right panel are a Coomassie-stained gel (top) and western blot probed with anti-Dsn1 antibodies (bottom) showing GST-CENP-T or GST immobilized on glutathione agarose in the presence of 6xHis-tagged Mis12 complex.

(B) CENP-C binds directly to the Mis12 complex in vitro. Western blot probed with the indicated antibodies showing the elution profile by size-exclusion chromatography for GST-CENP-C ΔC (amino acids 1–235) alone (top panel) or shifted in the presence of preassembled Mis12 complex (bottom panels). Arrows indicate the migration of standards with known Stokes radii: ferritin (61 Å), aldolase (48.1 Å), and ovalbumin (30.5 Å). Right figure is a Coomassie-stained gel showing GST-CENP-C ΔC or GST immobilized on glutathione agarose, alone or in the presence of the Mis12 complex.

(C) Percent sequence from the mass spectrometric analysis of one-step immunoprecipitations of either CENP-C or LAP^{GFP}-Mis12. Diverse samples prepared using identical conditions serve as negative controls for these purifications.

targeted to the mitochondrial outer membrane, mitochondria re-distributed to the mitotic spindle suggesting the presence of microtubule interactions (Figure S5B). Taken together, these data suggest that induced ectopic CENP-T foci can form interactions with microtubules.

To evaluate the interaction of CENP-T/CENP-C foci with microtubules, we assessed the segregation behavior of foci-containing chromosomes. Cell lines stably expressing the GFP-LacI control protein could be easily generated in U2OS-LacO cells. Control GFP-LacI expressing cells segregated the LacO-containing chromosome correctly in 90% of cases based on the number of foci in daughter cells (Figure 6C) and live-cell imaging (Figures 6D and 6E; Movie S1). In contrast, although we were able to generate stable GFP-CENP-T-ΔC-LacI cell lines in U2OS cells

lacking the LacO array, cell lines could only be generated in the presence of a LacO array if cells were grown in 1 mM IPTG to prevent binding of the LacI fusion protein to the LacO array. In cells transiently expressing GFP-CENP-T-ΔC-LacI, segregation of the LacO-containing chromosome was impeded, with the GFP-CENP-T-ΔC-LacI foci lagging behind the separating chromatin masses at anaphase (Figures 6D and 6E; Movie S2 and Movie S3), resulting in multiple foci in the daughter cells (Figure 6C). Similarly, CENP-C-ΔC-H2B or CENP-T-ΔC-H2B expressing cells showed rapid chromosome movement but failed to align chromosomes at a metaphase plate or complete a normal anaphase (Figures S3D and S3E, and Movie S4). Thus, the presence of ectopic CENP-T foci perturbs the segregation of chromosomes that contain an endogenous kinetochore.

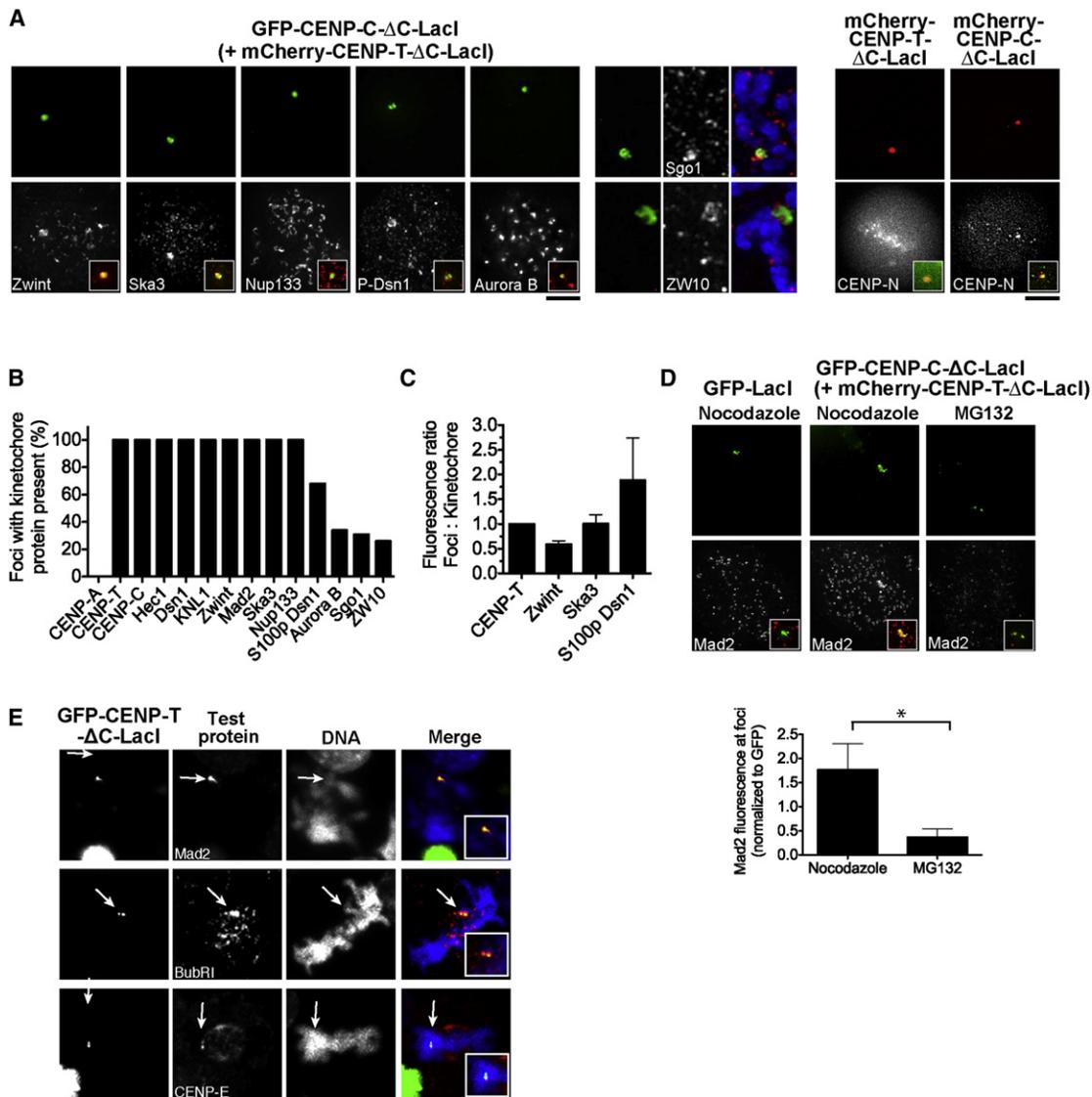


Figure 5. Induced CENP-T and CENP-C Foci Recruit Regulatory and Outer Kinetochore Proteins

(A) On the left are immunofluorescence images showing colocalization of kinetochore proteins with Lacl foci in cells transiently coexpressing mCherry-CENP-T-ΔC-Lacl (not shown) and GFP-CENP-C-ΔC-Lacl after 15 hr nocodazole treatment. Insets show a merge of kinetochore protein (red) and Lacl foci (green). Right panels show colocalization of GFP-CENP-N in cells expressing mCherry-CENP-T-ΔC-Lacl or mCherry-CENP-C-ΔC-Lacl.

(B) Quantification of the percentage of focus-containing cells with the indicated kinetochore protein at the ectopic site ($n \geq 20$).

(C) Quantification of the relative fluorescence of the indicated kinetochore proteins at kinetochores versus ectopic foci, in cells expressing Lacl fusion proteins. Quantification was done after 15 hr nocodazole treatment ($n \geq 10$ cells/condition, 20 kinetochores/cell, \pm SEM). Data are shown normalized to the foci/kinetochore ratio of the fusion protein.

(D) Top panels are immunofluorescence images showing colocalization of Mad2 with Lacl foci in cells transiently coexpressing mCherry-CENP-T-ΔC-Lacl (not shown) and GFP-CENP-C-ΔC-Lacl after 15 hr nocodazole treatment, or 1 hr treatment with MG132. Bottom panels show quantification of Mad2 fluorescence at ectopic foci after 15 hr nocodazole treatment or 1 hr treatment with MG132 ($n \geq 10$ cells/condition, 20 kinetochores/cell, \pm SEM). Asterisk indicates significant difference as determined by Mann Whitney U test ($p < 0.005$). Data are shown normalized to GFP fluorescence at ectopic foci.

(E) Immunofluorescence images showing colocalization of SAC proteins with Lacl foci in chicken DT40 cells containing a LacO array, and expressing GFP-CENP-T-ΔC-Lacl. Scale bar, 10 μ m.

Also see Figure S4.

Induced CENP-T-ΔC-Lacl Foci Can Direct Chromosome Segregation in the Absence of an Endogenous Kinetochore

To assess the function of an ectopic kinetochore focus in the absence of an endogenous kinetochore, we developed a centromere replacement system in chicken DT40 cells. We modified the chicken Z chromosome to insert LacO repeats \sim 50 kb from the endogenous Z centromere (Figures S5C and S5D), and placed loxP sites flanking the centromere region. Activation of Cre recombinase resulted in excision of the endogenous

mere replacement system in chicken DT40 cells. We modified the chicken Z chromosome to insert LacO repeats \sim 50 kb from the endogenous Z centromere (Figures S5C and S5D), and placed loxP sites flanking the centromere region. Activation of Cre recombinase resulted in excision of the endogenous

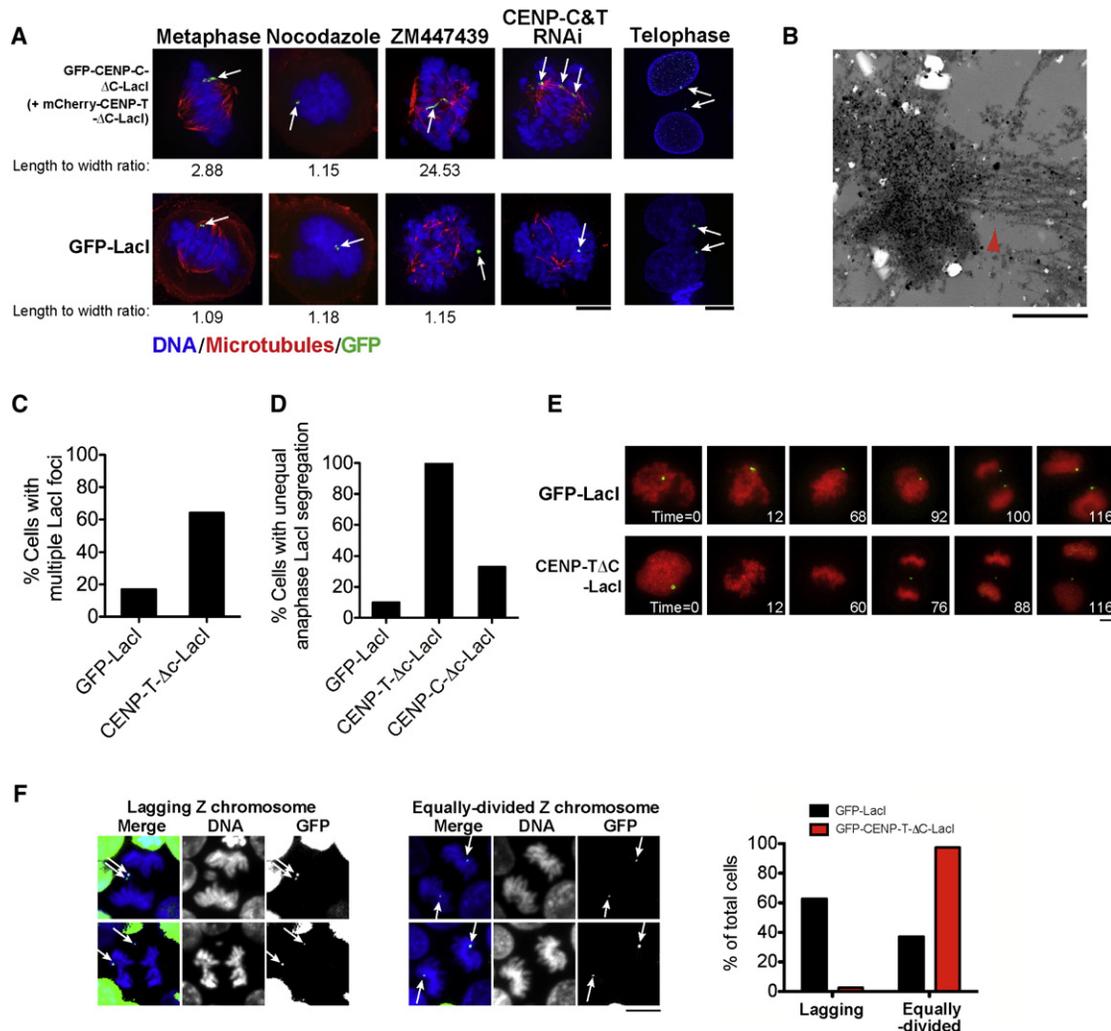


Figure 6. Induced CENP-T and CENP-C Foci Interact with Microtubules and Function in Chromosome Segregation

(A) Representative immunofluorescence images of cells expressing GFP-Lacl, or coexpressing RNAi-resistant mCherry-CENP-T- Δ C-Lacl (not shown) and GFP-CENP-C- Δ C-Lacl. Microtubules are shown in red. Cells were treated with 2 μ M ZM447439 or 3.3 μ M nocodazole, or 48 hr CENP-C and CENP-T RNAi as indicated. In all cases, cells were cold treated for 20 min prior to fixation to visualize stable kinetochore microtubule fibers. Arrows indicate the foci. The mean length/width ratio for the foci is shown below the indicated images ($n > 5$ cells).

(B) Electron micrographs of an ectopic CENP-T-Lacl focus showing the presence of microtubule attachments (red arrow). Dark spots indicate immunolabeling with anti-Hec1 antibody. These ectopic CENP-T-Lacl foci were defined by the size and extensive anti-Hec1 labeling of this structure relative to an endogenous kinetochore. Direct correlative light EM is shown to analyze ectopic kinetochore structure in Figure S5.

(C) Graph showing the percentage of cells with more than one Lacl foci 72 hr after expression of GFP-Lacl or GFP-CENP-T- Δ C-Lacl ($n = 200$ cells).

(D) Graph showing quantification of Lacl foci segregation in live cells expressing GFP-Lacl, GFP-CENP-T- Δ C-Lacl, or GFP-CENP-C- Δ C-Lacl.

(E) Selected images from time-lapse movies of U2OS-LacO cells expressing mCherry-histone-H2B to visualize chromatin, and GFP-CENP-T- Δ C-lacl or GFP-Lacl showing segregation of Lacl foci at anaphase. Time is shown in minutes after NEBD. Scale bars, 5 μ m.

(F) Centromere replacement assay in chicken DT40 cells (see Figure S6 for a schematic of this strategy). Left and middle panels are representative images of DT40 cells with GFP-Lacl fusion protein localized to a LacO array on the Z chromosome (arrows), after Cre recombinase mediated excision of the centromeric region of the same chromosome. Left panel shows representative images of the effected chromosome lagging at anaphase. Middle panel shows representative images of correct segregation of the Z chromosome. Right panel is a graph showing the percentage of GFP foci containing cells with lagging or equally dividing Z chromosomes 18 hr after addition of tamoxifen to induce excision of the endogenous centromere cells ($n = 78$ cells per condition). Also see Figure S5, and Movie S1, Movie S2, Movie S3, and Movie S4.

centromere (Shang et al., 2010), leaving only the LacO repeats (Figure S5E). We assessed the segregation behavior of the Z chromosome in the first division after centromere removal. Over 60% of cells expressing GFP-Lacl displayed lagging chromosomes following centromere excision (Figure 6F). In contrast,

in cells with GFP-CENP-T- Δ C-Lacl foci, lagging centromeres were observed in less than 3% of cells. Although expression of GFP-CENP-T- Δ C-Lacl does not rescue the long-term viability of these cells (most likely for technical reasons due to a lower frequency of foci formation), these data suggest that

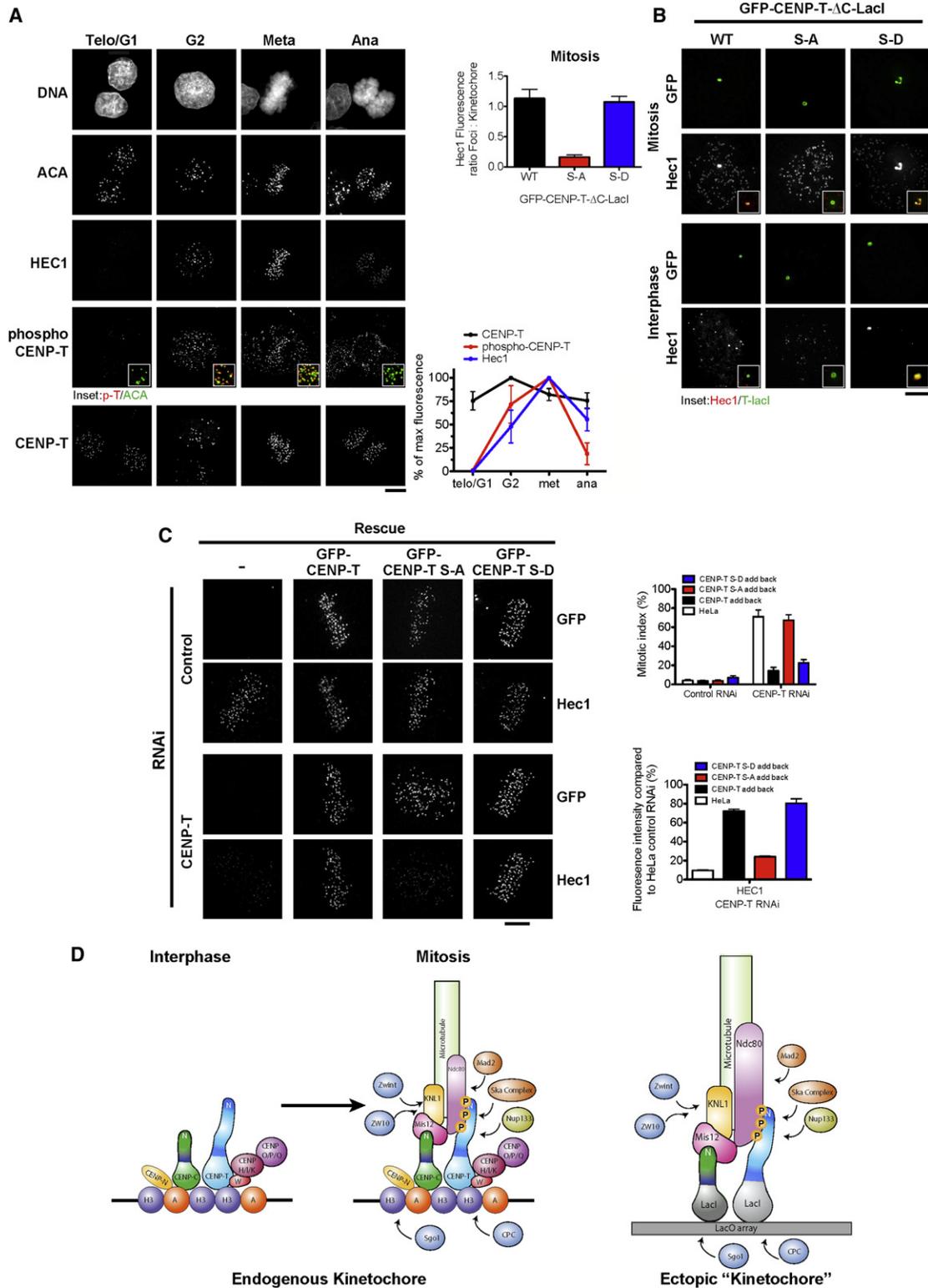


Figure 7. Mitotic Kinetochore Assembly Is Regulated by Phosphorylation of CENP-T by CDK

(A) Immunofluorescence images of HeLa cells at the indicated cell cycle stages. Right panel shows quantification of CENP-T, phospho-CENP-T, and Hec1 levels at centromeres ($n \geq 10$ cells/condition, 20 kinetochores/cell, \pm SEM). Scale bars, 5 μ m.

(B) Immunofluorescence images showing levels of Hec1/Ndc80 at ectopic foci in mitotic and interphase U20S LacO cells expressing LacI fusions of: GFP-CENP-T; GFP-CENP-T S-A (S or T to A mutations at amino acids 11, 27, 47, 85, and 195); or GFP-CENP-T S-D (S or T to D mutations at amino acids 11, 27, 85). Graph

CENP-T-induced ectopic foci are able to partially replace endogenous kinetochore function.

Phosphoregulation of Mitotic Kinetochore Assembly

Kinetochore assembly is a highly regulated process. Whereas CENP-C and CENP-T are present at the centromere throughout the cell cycle, the majority of outer kinetochore proteins are recruited only upon entry into mitosis. Because CENP-T is constitutively localized, but preferentially recruits the KMN network components during mitosis, we reasoned that these interactions might be regulated. An ideal candidate for controlling such interactions to distinguish mitosis from interphase functions is phosphorylation by cyclin-dependent kinase (CDK). In an ongoing proteomic analysis of kinetochore phosphorylation, and consistent with reports from proteome-wide phosphorylation studies (Dephoure et al., 2008; Nousiainen et al., 2006; Santamaria et al., 2011), we found that CENP-T is phosphorylated on multiple potential CDK consensus sites in HeLa cells (Figure S6A). In addition, mass spectrometry analysis of recombinant CENP-T/W complex demonstrated that CDK directly phosphorylates these sites *in vitro* (Figure S6A).

To analyze the dynamics of CENP-T phosphorylation, we generated an antibody specific to CENP-T phosphorylated at one CDK site (serine 47; Figure S6B). Immunostaining of HeLa cells indicated that CENP-T becomes phosphorylated in G2, concomitant with the localization of Hec1 to centromeres (Figure 7A). CENP-T remained phosphorylated until anaphase, when fluorescence levels dropped to ~20% of their metaphase maximum, consistent with a decrease in CDK activity at this time. In contrast, Hec1 levels remained relatively high at anaphase, and were not reduced until telophase, consistent with additional regulatory mechanisms for kinetochore disassembly.

To test the role of CENP-T phosphorylation in controlling mitotic kinetochore assembly, we mutated the CDK phosphorylation sites at the N terminus of CENP-T to alanine to prevent phosphorylation, and to aspartate to mimic constitutive phosphorylation. Preventing CENP-T phosphorylation abolished the targeting of Ndc80/Hec1 to ectopic CENP-T-Lacl foci during both interphase and mitosis (Figure 7B). Preventing phosphorylation of CENP-T also disrupted the targeting of Dsn1 to ectopic foci during mitosis, although Dsn1 continued to localize to foci in a subset of interphase cells (data not shown). In contrast, constitutively mimicking CENP-T phosphorylation results in the targeting of Ndc80/Hec1 to the majority of both interphase and mitotic ectopic foci (Figure 7B; data not shown).

In addition to regulating assembly of ectopic CENP-T foci, these phosphorylation sites are critical for controlling the function of endogenous kinetochores. Eliminating CENP-T phos-

phorylation prevented Ndc80/Hec1 localization and caused defects in chromosome segregation that result in a mitotic arrest (Figure 7C). Similarly, expression of phosphoinhibitory CENP-T in chicken DT40 cells lacking endogenous CENP-T greatly reduced Ndc80/HEC1 levels at kinetochores as well as cell viability (Figures S6C and S6D). However, in the context of the endogenous kinetochore, phosphomimetic CENP-T did not cause constitutive kinetochore assembly (Figure 7C). Similarly, treatment of mitotic cells with a CDK inhibitor caused a rapid loss of CENP-T phosphorylation at kinetochores but did not cause premature disassembly of Hec1 (data not shown), suggesting that additional factors likely contribute to kinetochore disassembly.

Taken together, these data demonstrate that in the absence of CENP-A, CENP-C and CENP-T are sufficient to direct the assembly of mitotically regulated kinetochore-like structures (Figure 7D). Thus, CENP-C and CENP-T can provide a regulated platform for mitotic kinetochore assembly in vertebrate cells.

DISCUSSION

The observations described here reveal that two key DNA-binding kinetochore components, CENP-C and the CENP-T/W complex, can function to direct kinetochore formation in vertebrate cells in the absence of the specialized centromeric CENP-A nucleosomes. Rather than relying on protein depletion to assess the requirement of proteins in kinetochore assembly, we have developed an ectopic-targeting assay to analyze the sufficiency of kinetochore components for assembly of this structure. Our results demonstrate that, although ectopic localization of CENP-A results in mislocalization of a small subset of kinetochore proteins, ectopic localization of CENP-T and CENP-C results in recruitment of multiple additional proteins, including the microtubule-binding KMN network. These structures are at least partially functional based on the stoichiometric recruitment of diverse outer kinetochore proteins to ectopic CENP-T and CENP-C foci, evidence of interactions with microtubules, the ability of the foci to direct chromosome segregation, and the proper regulation of mitotic kinetochore assembly. Taken together, these data indicate that the presence of CENP-C and CENP-T/W can provide a platform for outer kinetochore assembly in vertebrate cells (Figure 7D).

CENP-T/W and CENP-C Form a Platform for Kinetochore Assembly in Vertebrate Cells

Centromere proteins play two important, yet distinct, functions in the recruitment of kinetochore proteins to chromatin during mitosis: specifying the site of kinetochore formation on the chromosome, and assembling the protein architecture of this

shows the relative fluorescence of Hec1/Ndc80 at kinetochores versus ectopic foci, in mitotic cells expressing the indicated CENP-T Lacl fusion proteins. Quantification was done after 15 hr nocodazole treatment ($n \geq 10$ cells/condition, 20 kinetochores/cell, \pm SEM). Data are shown normalized to the foci/kinetochore ratio of CENP-T.

(C) Left panels are immunofluorescence images showing levels of Hec1/Ndc80 48 hr after RNAi depletion of CENP-T in HeLa cells or HeLa cell lines expressing RNAi-resistant GFP-CENP-T, GFP-CENP-T S-A, or GFP-CENP-T S-D. Right panels show quantification of mitotic index and Hec1/Ndc80 levels, after RNAi depletion of CENP-T and add back of the indicated GFP-CENP-T proteins (\pm SEM).

(D) Model depicting interactions at endogenous and ectopic kinetochores.

Also see Figure S6.

structure. Previous work demonstrated that CENP-A is essential for specifying the site of kinetochore formation. However, whether CENP-A plays a direct molecular role in kinetochore assembly was unclear because data on its sufficiency for kinetochore formation varied among eukaryotes. In *Drosophila* the presence of ectopic CENP-A foci is sufficient for kinetochore formation (Heun et al., 2006). This contrasts with our work in human cells, in which ectopic localization of CENP-A is sufficient to mislocalize only a small subset of kinetochore proteins. Thus, in human cells CENP-A is important for specifying the site of kinetochore formation but is not sufficient for assembly of this structure. Instead of functioning itself to direct kinetochore assembly, the specialized CENP-A nucleosomal structure may be required to generate an environment that is permissive for kinetochore assembly, or that provides structural rigidity to this chromosomal region.

We have defined an essential role for the CCAN proteins CENP-C and CENP-T (the latter of which is absent in *Drosophila*) (Cheeseman and Desai, 2008) in directing kinetochore assembly in vertebrate cells (this work; Hori et al., 2008). Ectopic localization of CENP-A in human cells does not cause recruitment of CENP-T/W, explaining at least in part why CENP-A alone is not sufficient for kinetochore assembly. Consistent with this, we previously found that the CENP-T/W complex coimmunoprecipitates with H3, but not CENP-A containing nucleosomes (Hori et al., 2008). Thus, our results demonstrate that additional proteins, including CENP-T, are essential factors for kinetochore formation and can act independently of CENP-A to drive kinetochore assembly (Figure 7C). Defining the mechanism by which the DNA-binding activity of the CENP-T/W complex targets these proteins to centromeres represents a goal for future work.

Our finding that the KMN network proteins are recruited to kinetochores by CENP-C and CENP-T provides insight into the mechanism by which the microtubule-binding activity of the kinetochore assembles onto this structure. Based on our analysis of CENP-C and CENP-T depletion, CENP-C and CENP-T activity in induced ectopic kinetochore assembly, and the protein interactions defined by our biochemical analysis, both CENP-C and CENP-T can act directly to recruit components of the KMN network. In addition electron microscopy analysis of kinetochore proteins in chicken DT40 cells indicates that the N terminus of CENP-T colocalizes with outer kinetochore components such as Ndc80 during mitosis, supporting an interaction between these proteins (Suzuki et al., 2011). CENP-C and CENP-T can directly or indirectly recruit multiple additional outer kinetochore proteins to ectopic foci based on our analysis of selected subunits. Thus, CENP-C and CENP-T can provide a platform for kinetochore assembly.

Phosphoregulation of Mitotic Kinetochore Assembly

Of the almost 100 vertebrate kinetochore proteins, only 17 are constitutively present at centromeres. During a period of less than 2 hr during a 24 hr cell cycle in human cells, more than 60 mitotic-specific kinetochore proteins must be assembled onto this platform, and then subsequently be removed from these sites. However, very little is known about the mechanisms that control this highly ordered assembly process. Here, we

demonstrate that phosphorylation of CENP-T by CDK can control kinetochore assembly (Figure 7). Although the regulation of mitotic kinetochore assembly is likely to be a multistep process, CENP-T regulation in controlling the kinetochore assembly state represents an important step and provides additional support for the central role of CENP-T as a key kinetochore assembly factor.

Toward Sequence-Dependent Vertebrate Kinetochores

We have demonstrated that foci generated by the artificial tethering of CENP-C and CENP-T to ectopic loci can participate in chromosome segregation, and can at least partially complement removal of the endogenous centromere. This suggests the possibility that such a system could be used to generate human artificial chromosomes (HACs). Previous attempts to generate HACs have been hampered by difficulties generating a functional centromeric region, which is thus far only possible in a small subset of cell lines (Harrington et al., 1997; Masumoto et al., 1998; Ohzeki et al., 2002). The system we describe here bypasses both the requirement for alpha-satellite sequences and CENP-A and has the potential to mediate chromosome segregation simply by tethering CENP-C and CENP-T to a chromatin locus, for example using a LacO/LacI system such as the one described here, or a tetO/tetR system as used by Nakano et al. (2008), where such synthetic kinetochores could be turned on by addition of tetracycline.

However, although ectopic targeting of CENP-T and CENP-C directs formation of kinetochore-like structures, there are still critical differences between the LacO-based kinetochore foci in U2OS cells and endogenous kinetochores. First, there are several proteins that we did not observe at the induced CENP-T/CENP-C foci under the tested conditions in human cells. This may reflect technical issues (such as the nature of the available antibodies) because some proteins such as CENP-E were observed at ectopic foci in chicken DT40 cells, but not human cells. Alternatively, this may suggest that either CENP-A or the C termini of CENP-T or CENP-C are required to recruit some kinetochore proteins. Second, although endogenous kinetochores are of a homogenous size in vertebrate cells based on fluorescent intensity, the ectopic kinetochore-like structures are typically 5- to 50-fold larger. This is due at least in part to the extended array of LacO repeats that directs formation of these foci. Endogenous kinetochore size is homogeneous, even though the size of alpha-satellite centromeric chromatin can vary, suggesting epigenetic regulation of kinetochore size is critical. Testing different sized LacO repeats or modulating the expression level of CENP-T and CENP-C may provide a way to generate structures more similar in size to endogenous kinetochores. Third, although we have bypassed the requirement for CENP-A in directing the recruitment of outer kinetochore proteins, it is important to note that centromeric chromatin may be critical to proper kinetochore function. Centromeric chromatin structure may reflect a role for CENP-A, or the specialized histone modifications that are found at centromeres, neither of which is likely to be present at the ectopic foci. This chromosome structure may be required to resist spindle forces or prevent chromosomal twisting that would result in merotelic attachments. Indeed, we found that some ectopic

kinetochore-like structures were highly deformed and showed apparent merotelic attachments to the spindle.

In sum, although CENP-A is essential for specifying the site of kinetochore formation, our studies reveal that the CCAN proteins CENP-T and CENP-C act as key components to drive assembly of a functional kinetochore capable of binding microtubules to allow accurate chromosome segregation during mitosis.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection

Human cell lines were maintained as described previously (Kline et al., 2006). The U2OS LacO cell line (a generous gift from S. Janicki) was maintained in 200 μ g/ml Hygromycin B. Stable clonal cell lines expressing GFP^{LAP} fusions were generated in HeLa cells as described previously (Cheeseman et al., 2004). Codon-optimized and RNAi-resistant CENP-T cDNA (Mr. Gene) was used in all experiments. Transient transfections were carried out using Effectene (QIAGEN) according to the manufacturer's instructions. Pools of siRNAs against CENP-T (CAAGAGAGCAGUUGCGGCA, GACGAUAGCCA GAGGGCGU, AAGUAGAGCCCUUACACGA, CGGAGAGCCCUUUGAAAA) and CENP-C (GCGAAUAGAUUAUCAAGGA, GAACAGAAUCCAUCACAAA, CGAAGUUGAUAGAGGAUGA, UCAGGAGGAUUCGTGAUUA), and a nontargeting control were obtained from Dharmacon. RNAi experiments were conducted as described previously (Kline et al., 2006).

Immunofluorescence and Microscopy

Immunofluorescence in human cells was conducted as described previously (Kline et al., 2006), using antibodies described in Table S1. Cy2-, Cy3-, and Cy5-conjugated secondary antibodies were obtained from Jackson Laboratories. DNA was visualized using 10 μ g/ml Hoechst. Where indicated, cells were incubated for 15 hr with 3.3 μ M nocodazole, for 1 hr with 20 μ M MG132, or for 1 hr with 2 μ M ZM447439.

Images were acquired on a DeltaVision Core deconvolution microscope (Applied Precision) equipped with a CoolSnap HQ2 CCD camera. Approximately 40 Z-sections were acquired at 0.2 μ m steps using a 100 \times , 1.3 NA Olympus U-PlanApo objective. Images were deconvolved using the DeltaVision software. For time-lapse imaging, cells were maintained in CO₂-independent media (Invitrogen) at 37°C, and imaged every 4 min using eight Z-sections at 0.8 μ m intervals using a 40 \times Olympus UApo/340 objective. To quantify fluorescent intensity, individual kinetochores and ectopic foci were analyzed as described previously (Kline et al., 2006).

Protein Purification and Biochemical Assays

For the expression and purification of the recombinant proteins in *E. coli*, 6xHis-CENP-T (full length or amino acids 288–561) and untagged CENP-W were coexpressed from pST39. 6xHis-Mis12 complex and Ndc80^{Bonsai} complex were expressed as described previously (Kline et al., 2006; Ciferri et al., 2008). GST-CENP-C N terminus (amino acids 1–235), GST-CENP-T, and GST-CENP-K were expressed from pGEX-6P-1. Proteins were purified using Glutathione agarose (Sigma) or Ni-NTA agarose (QIAGEN) according to the manufacturer's guidelines and then exchanged into 25 mM HEPES (pH 7.5), 200 mM NaCl, 1 mM EDTA, 1 mM β -mercaptoethanol, followed by size-exclusion chromatography. For in vitro-binding assays, proteins were bound to Glutathione agarose or Ni-NTA agarose, incubated with test proteins, washed three times with PBS, 250 mM NaCl, and 0.1% Tween, 1 mM β -mercaptoethanol, or 50 mM HEPES (pH 7.5), 300 mM NaCl, 0.1% Tween, 25 mM Imdazole, and resuspended in SDS-PAGE sample buffer.

GFP^{LAP}-tagged Mis12 complex and endogenous CENP-C were isolated from HeLa cells as described previously (Cheeseman, 2005). CENP-C was isolated using rabbit anti-CENP-C antibodies. Proteins were identified by mass spectrometry using an LTQ XL Ion trap mass spectrometer (Thermo) using MudPIT and SEQUEST software as described previously (Washburn et al., 2001).

Chicken DT40 Experiments

DT40 cells were cultured and transfected as described previously (Hori et al., 2008). Mutant or full-length CENP-C and CENP-T constructs under control of a tetracycline-repressive promoter were transfected into DT40 cells with a tet-repressible transactivator in the presence of tetracycline.

Immunofluorescent staining of chicken cells was performed as described previously (Fukagawa et al., 2001), using antibodies described in Table S1. Images were collected with a cooled EM CCD camera (QuantEM, Roper Scientific Japan) mounted on an Olympus IX71 inverted microscope with a 100 \times objective lens together with a filter wheel and a DSU confocal unit. Approximately 20 Z-sections were acquired at 0.3 μ m steps.

DT40 cells in which the centromere of chromosome Z can be conditionally removed were used in centromere replacement assays (Shang et al., 2010). A LacO repeat was inserted at an \sim 50 kb region adjacent to the centromere by homologous recombination. GFP-CENP-T Δ C-LacI or GFP-LacI constructs were introduced into these cells. Using a Mer-Cre-Mer construct integrated in these cells, Cre recombinase was activated upon hydroxytamoxifen (OHT) addition. Removal of the centromere was confirmed by Southern hybridization (Figure S5). Cells with lagging chromosomes during anaphase were counted 18 hr after addition of OHT.

Electron Microscopy

U2OS cells were treated with 500 ng/ml nocodazole or 10 μ M MG132 for 2 hr to prepare mitotic cells. Mitotic cells were cyto-centrifuged onto a glass slide, permeabilized by 1% Triton, and fixed in 3% paraformaldehyde/1.5% glutaraldehyde for 15 min. Samples were rinsed in 0.5% BSA/0.1% Triton in PBS, incubated for 1 hr at 37°C with primary antibodies (anti-CENP-T or anti-Hec1 (9G3)), washed three times in 0.5% BSA/0.1% Triton in PBS, and incubated with FITC-conjugated secondary antibodies and 1.4 nm gold-labeled secondary antibodies (Nanoprobes), simultaneously for 2 hr at 37°C. Samples were observed by fluorescence microscopy to identify CENP-T-Lac-I-GFP containing foci. The position of the chromosome with CENP-T-Lac-I-GFP was marked, and samples were fixed with 2.5% glutaraldehyde/3% PFA in 0.1 M cacodylate buffer (pH 7.2), at 4°C for 20 hr. Samples were silver enhanced using a HQ Silver kit (Nanoprobes) according to the manufacturer's protocol. Postfixation was performed in 0.5% OsO₄ on ice. The cells were dehydrated in ethanol and then infiltrated with Epon812. Polymerization was performed at 60°C for 48 hr. Serial sections were cut with an ultramicrotome equipped with a diamond knife (170 nm). Samples were stained by uranyl acetate and lead citrate and imaged at room temperature using a JEM1010 TEM (JEOL) at 100 kV.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures, one table, and four movies and can be found with this article online at [doi:10.1016/j.cell.2011.03.031](https://doi.org/10.1016/j.cell.2011.03.031).

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