Induced Ectopic Kinetochore Assembly Bypasses the Requirement for CENP-A Nucleosomes

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SUMMARY

Accurate chromosome segregation requires assembly of the multienzyme 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
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 kinetochore microtubule-binding proteins and how kinetochore assembly is regulated to recruit outer kinetochore components during mitosis is a key goal.

To define the mechanisms that direct kinetochore assembly downstream of CENP-A, we analyzed the constitutive kinetochore components, focusing on the DNA-binding proteins CENP-C and the CENP-T/W complex. We find that ectopic targeting of CENP-C and CENP-T can generate foci that function as kinetochore-like structures in the absence of CENP-A, demonstrating that CENP-C and the CENP-T/W complex function as core kinetochore assembly factors in vertebrate cells.

**RESULTS**

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

Specialized centromeric CENP-A nucleosomes are thought to provide the foundation for kinetochore assembly. To test the sufficiency of CENP-A for directing kinetochore 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 kinetochore proteins tested (Figure 1B; see Figure S1 available online). Thus, in human cells, mistargeting of CENP-A does not result in ectopic kinetochore-like structures in the absence of CENP-A, demonstrating that CENP-C and the CENP-T/W complex function as core kinetochore assembly factors in vertebrate cells.
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 Kinetochore 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 tested, including representative components of the microtubule-binding KMN network—KNL1, Dsn1 (Mis12 complex), and Ndc80/Hec1 (Figure 1E), as well as the inner centromere protein Aurora B kinase (data not shown). However, CENP-C and CENP-T display potential 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 Kinetochore 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 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.
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-DΔC or GFP-CENP-T-DΔC fusions, endogenous CENP-A, CENP-T, and CENP-C did not co-localize 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-DΔC and GFP-CENP-T-DΔC fusions, but not with GFP controls (Figure 3D; Figure S3B). We also observed colocalization of Ndc80 and Nu2 with GFP-CENP-T-DΔC-LacI fusions in chicken DT40 cells (Figure S3F). Importantly, simultaneous expression of mCherry-CENP-T-DΔC and GFP-CENP-C-DΔC 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-DΔC fusions preferentially recruited Dsn1 and KNL1, whereas CENP-T-DΔC-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 (Ndc80Bonsai; 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 Przewloka 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-DΔC-LacI and GFP-CENP-T-DΔC-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 phosphate-body 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-DΔC-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-DΔC-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-DΔC-LacI focus was dependent on...
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 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-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-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-LacI or GFP-CENP-C-LacI, or coexpressing mCherry-CENP-T-LacI (not shown) and GFP-CENP-C-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.
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 interac-
tions with microtubules.

To evaluate the interaction of CENP-T/CENP-C foci with 
microtubules, we assessed the segregation behavior of foci-con-
taining 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-con-
taining 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-D LacI cell lines in U2OS cells 
lacking the LacO array, cell lines could only be generated in the presence of an 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-D LacI, segregation 
of the LacO-containing chromosome was impeded, with the 
GFP-CENP-T-D 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-D H2B or CENP-T-D 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 segrega-
tion of chromosomes that contain an endogenous kinetochore.

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-Ndc80Bonsai, 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-Ndc80Bonsai 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 LAPGFP-Mis12. Diverse samples prepared using identical conditions serve as negative controls for these purifications.
Induced CENP-T-ΔC-LacI 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 (~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 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 LacI foci in cells transiently coexpressing mCherry-CENP-T-ΔC-LacI (not shown) and GFP-CENP-C-ΔC-LacI after 15 hr nocodazole treatment. Insets show a merge of kinetochore protein (red) and LacI foci (green). Right panels show colocalization of GFP-CENP-N in cells expressing mCherry-CENP-T-ΔC-LacI or mCherry-CENP-C-ΔC-LacI.
(B) Quantification of the percentage of focus-containing cells with the indicated kinetochore protein at the ectopic site (n ≥ 20).
(C) Quantification of the relative fluorescence of the indicated kinetochore proteins at kinetochores versus ectopic foci, in cells expressing LacI fusion proteins. Quantification was done after 15 hr nocodazole treatment (n ≥ 10 cells/condition, 20 kinetochores/cell, ±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 LacI foci in cells transiently coexpressing mCherry-CENP-T-ΔC-LacI (not shown) and GFP-CENP-C-ΔC-LacI 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 ≥ 10 cells/condition, 20 kinetochores/cell, ±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 LacI foci in chicken DT40 cells containing a LacO array, and expressing GFP-CENP-T-ΔC-LacI. Scale bar, 10 μm.

Also see Figure S4.
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-LacI displayed lagging chromosomes following centromere excision (Figure 6F). In contrast, in cells with GFP-CENP-T-ΔC-LacI foci, lagging centromeres were observed in less than 3% of cells. Although expression of GFP-CENP-T-ΔC-LacI 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

centrionome (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-LacI displayed lagging chromosomes following centromere excision (Figure 6F). In contrast,
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 ≥ 10 cells/condition, 20 kinetochores/cell, ±SEM). Scale bars, 5 μm.

(B) Immunofluorescence images showing levels of Hec1/Ndc80 at ectopic foci in mitotic and interphase U2OS 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

(C) Rescue

(D) Interphase Mitosis

Endogenous Kinetochore Ectopic “Kinetochore”
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 phosphorylation 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
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 chromosomal heterochromatin 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 μg/ml Hygromycin B. Stable clonal cell lines expressing GFP-LAP fusions were generated in HeLa cells as described previously (Cheseman 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 (CAAGAGACCGAGUGCGGCA, GACGAAUAGCCA GAGGGCGU, AAGUAGAGCCUUACACGA, CGGAGAGCCCUGCUUGAAA) and CENP-C (GCGAAUAGAUUAUACGGA, GAAACAGAAUUCACCAAAAA, CGAAUGUGUAAGGGAUGA, UCAGGAGAUAUCGTGAUA), 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 μM DAPI. Images were acquired at 0.2 μm steps using a 100

**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.

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SUPPLEMENTAL REFERENCES


Figure S1. Multiple Kinetochore Proteins Are Not Mislocalized by CENP-A Overexpression, Related to Figure 1B

(A) Representative images of kinetochore components not mis-localized by CENP-A overexpression. Dsn1, KNL1, ZW10, Nup133, CENP-E, Aurora B, MCAK, Bub1, and BubR1 are visualized by immunofluorescence. CENP-O is visualized in a HeLa cell line expressing GFP-LAP-CENP-O.

(B) Immunofluorescence images showing co-overexpression of CENP-A and CENP-C, resulting in the mistargeting of Dsn1 to chromosome arms.
Figure S2. CENP-T Is Not an Oligomer, Related to Figure 2

(A) Coomassie-stained gel showing the profile of the CENP-T/W complex in a 5%–20% sucrose gradient. Arrows indicate the migration of standards with known S values: RNase (2.6), BSA (4.3), Aldolase (7.35). CENP-T/W shows an S value of ~3.8 indicating a native molecular weight of ~93 kDa, compared to a predicted molecular weight of 71 kDa for a heterodimer, suggesting the complex does not exist as an oligomer.

(B) Representative image of a cell showing overexpression of GFP-CENP-T. Overexpression does not cause mislocalization to chromosome arms.
Figure S3. Targeting of CENP-C and CENP-T to Chromosome Arms Causes Mislocalization of the KMN Network and Chromosome Segregation Defects, Related to Figure 3

(A) Immunofluorescence images showing centromeric localization of DNA binding kinetochore proteins in cells transiently expressing GFP-CENP-TΔC-H2B or GFP-CENP-CΔC-H2B.

(B) Immunofluorescence images showing mis-localization of KMN network proteins to chromosome arms in cells transiently expressing GFP-CENP-TΔC-H2B or GFP-CENP-CΔC-H2B, or cells coexpressing mCherry-CENP-TΔC-H2B (not shown) and GFP-CENP-CΔC-H2B.

(C) Left, images showing centromeric localization of the indicated kinetochore proteins in cells expressing GFP-H2B as a control. Right, immunofluorescence images showing mis-localization of the indicated proteins to chromosome arms in cells transiently coexpressing mCherry-CENP-TΔC-H2B (not shown) and GFP-CENP-CΔC-H2B.

(D) Graph showing quantification of the stages of mitosis in cells expressing GFP-CENP-TΔC-H2B and GFP-CENP-CΔC-H2B, individually or in combination. N = 100 cells/condition.

(E) Left, graph showing quantification of the duration of mitosis from Nuclear Envelope Breakdown (NEBD) to chromatin decondensation in cells expressing GFP-H2B, GFP-CENP-TΔC-H2B, or GFP-CENP-CΔC-H2B, or coexpressing mCherry-CENP-TΔC-H2B (not shown) and GFP-CENP-CΔC-H2B. N > 50 cells per...
condition. Bar indicates the mean. Middle, selected images from time-lapse movies of HeLa cells coexpressing mCherry-CENP-T-ΔC-H2B (not shown) and GFP-CENP-C-ΔC-H2B. Top panel shows mitotic arrest, bottom panel shows accelerated mitosis. Time is indicated in min after NEBD. Right, graph showing quantification of the mitotic exit phenotype in cells expressing GFP-H2B, GFP-CENP-T-ΔC-H2B, or GFP-CENP-C-ΔC-H2Bm or coexpressing mCherry-CENP-T-ΔC-H2B (not shown) and GFP-CENP-C-ΔC-H2Bm. N > 50 cells. Scale bars, 5 μm.

(F) Images of chicken DT40 cells containing a LacO array, expressing the indicated LacI fusion proteins. Ndc80 and Nuf2 are visualized by immunofluorescence. Scale bar, 10 μm.
Figure S4. Several Kinetochore Proteins Are Not Mislocalized by CENP-T or CENP-C Mistargeting, Related to Figure 5

(A) Immunofluorescence images showing only centromeric localization of the indicated proteins in cells expressing GFP-LacI, or localization of GFP-CENP-N in cells expressing mCherry-LacI.

(B–F) Immunofluorescence images of kinetochore components not mis-localized by mCherry-CENP-T-ΔC-H2B and GFP-CENP-C-ΔC-H2B, or mCherry-CENP-T-ΔC-LacI and GFP-CENP-C-ΔC-LacI. CENP-E, ZW10, Bub1, MCAK, and BubR1 are visualized by immunofluorescence, CENP-O is visualized using a HeLa cell line expressing GFP LAP-CENP-O, and Mis18 is visualized by cotransfection with GFP-Mis18. Scale bar, 5 μm.
Figure S5. Ectopic Foci Interact with Microtubules, Related to Figure 6

(A) Chromosome spreads observed by electron microscopy (EM). Left panels shown fluorescence microscope images of a control chromosome, and corresponding EM image of the same chromosome. Red signals were visualized by anti-CENP-T antibody. Right panels show fluorescence microscope images of a CENP-T-LacI foci-containing chromosome and corresponding EM image. A primary constriction-like structure was observed at the position where CENP-T accumulated. The length of the structure is ~1.5 μm relative to ~0.3 μm for an endogenous kinetochore. Scale bars: IF, 10 μm; EM, 1 μm.

(B) CENP-T was targeted to the cytoplasmic surface of the mitochondrial outer membrane using a “mito” tag (Bear et al., 2000). Representative immunofluorescence images showing colocalization of Ndc80/Hec1, with GFP-CENP-T-D-mito, but not with GFP-CENP-T-D-mito with 5 CDK phosphorylation sites mutated to alanine (S-A). Scale bar, 5 μm.

(C and D) Schematic representation of strategies used to remove the centromeric region of the Z chromosome in chicken DT40 cells, and to generate an ectopic LacO/LacI foci on the same chromosome.

(E) Southern blot analysis of genomic DNA from DT40 cells expressing GFP-LacI or GFP-CENP-T-D-LacI showing similar levels of excision in each case. DNA was digested with Aff II and probed for the bleomycin cassette. The presence of a 17.3 kb fragment indicates excision of the centromeric region.
Figure S6. Recruitment of Ndc80/HEC1 to Kinetochores Is Regulated by Phosphorylation of CENP-T, Related to Figure 7

(A) Protein sequence of human and chicken CENP-T, with mapped CDK phosphorylation sites represented in the indicated colors.

(B) Western blot showing recombinant CENP-T, alone or after 1 hr incubation with recombinant CDK2/cyclin A. Left panel is probed with anti-CENP-T antibody, right panel is probed with anti-phosphoS47-CENP-T antibody.

(C) Representative immunofluorescence images of chicken DT40 cells after depletion of endogenous CENP-T and tetracycline induced expression of mutant CENP-T with S or T to A or D mutations at amino acids 16, 24, 56, 72, 88 and 325. Scale bar, 10 μm.

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