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Premitotic Assembly of Human CENPs -T and -W Switches Centromeric Chromatin to a Mitotic State

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Abstract

Centromeres are differentiated chromatin domains, present once per chromosome, that direct segregation of the genome in mitosis and meiosis by specifying assembly of the kinetochore. They are distinct genetic loci in that their identity in most organisms is determined not by the DNA sequences they are associated with, but through specific chromatin composition and context. The core nucleosomal protein CENP-A/cenH3 plays a primary role in centromere determination in all species and directs assembly of a large complex of associated proteins in vertebrates. While CENP-A itself is stably transmitted from one generation to the next, the nature of the template for centromere replication and its relationship to kinetochore function are as yet poorly understood. Here, we investigate the assembly and inheritance of a histone fold complex of the centromere, the CENP-T/W complex, which is integrated with centromeric chromatin in association with canonical histone H3 nucleosomes. We have investigated the cell cycle regulation, timing of assembly, generational persistence, and requirement for function of CENPs -T and -W in the cell cycle in human cells. The CENP-T/W complex assembles through a dynamic exchange mechanism in late S-phase and G2, is required for mitosis in each cell cycle and does not persist across cell generations, properties reciprocal to those measured for CENP-A. We propose that the CENP-A and H3-CENP-T/W nucleosome components of the centromere are specialized for centromeric and kinetochore activities, respectively. Segregation of the assembly mechanisms for the two allows the cell to switch between chromatin configurations that reciprocally support the replication of the centromere and its conversion to a mitotic state on postreplicative chromatin.

Introduction

The centromere is the genetic locus present in a single copy on each eukaryotic chromosome that provides the transmission function of the genome across mitotic and meiotic generations [1,2]. An epigenetically determined locus, it functions by directing assembly of the kinetochore in mitosis and meiosis, a dynamic protein complex that possesses microtubule binding and motor activities as well as spindle assembly checkpoint complexes [3,4]. The centromere is unique in that, in almost all species, its identity is not deterministically related to the DNA sequence that underlies it [5,6]. This has been dramatically underscored by the discovery that certain centromeres of the genus *Equus* reside on unique sequence DNA [7,8]. Rather, centromere identity seems to be specified at the chromatin level, through a distinctive population of nucleosomes made with CENP-A or cenH3, a centromere-specific histone H3 variant found in all eukaryotes [9–12].

The composition and molecular organization of CENP-A nucleosomes and their mechanistic contribution to centromere determination in several organisms has been a subject of intensive investigation and debate [13,14]. Cse4, the CENP-A of budding yeast, has been reported to form classical octameric nucleosome core complexes with histones H4, H2A, and H2B [15], tetrameric half-nucleosomes [16], and other complexes [17]. Distinctive structural organization within a CENP-A:H4 tetrameric core [18], unusual mechanical rigidity of the nucleosome [19], and a right-handed winding of DNA, opposite that of conventional nucleosomes [20] have been proposed as critical molecular features that could be involved in maintenance of centromere identity. These features are thought to function, in part, to coordinate a specific, multistep chromatin assembly pathway that initiates in anaphase/telophase in human cells and continues throughout G1 [21–26]. However, CENP-A is unlikely to be the sole determinant of centromere identity, as misincorporation of CENP-A only rarely results in ectopic centromere formation [27,28].

A large group of additional proteins assemble on centromeric chromatin in pathways that are dependent on CENP-A and feed back to influence its assembly (interphase centromere complex [ICEN]) [29]; CENP-A nucleosome associated complex, CENP-A distal complex [NAC/CAD] [30]; CENP-H/I complex [31]. Collectively known as the constitutive centromere associated network (CCAN) [32], functional examination of the role of these proteins in vertebrate centromere propagation and kinetochore formation has revealed a complex network of interdependent
Author Summary

The centromere is a strange locus that derives its identity from the proteins that shape it rather than the DNA sequences it contains. It also functions in a remarkably singular way, providing a motor and command control center for the chromosome in conjunction with the kinetochore. Key to centromere identity is the chromatin that comprises it, which has a unique nucleosomal “bead on a string” including a special centromeric histone H3, called CENP-A. Found in alternating clusters of nucleosomes with “regular” histone H3, CENP-A is crucial for propagating centromere identity as well as for regulating kinetochore function. In this study, we have analyzed the cell cycle dynamics of CENP-T and CENP-W, another two components of the constitutive centromere associated network. We showed that, unlike CENP-A, CENP-T/W are not inherited stringently by daughter cells. Instead, these complexes - which are bound to the interstitial “regular” H3 nucleosome domains - assemble after DNA replication and are required for kinetochore formation. Thus, we propose that a stable CENP-A nucleosome population plays a role in centromere locus inheritance to daughter cells, while dynamic CENP-T/W and H3 nucleosomes provide a cycling function that triggers kinetochore assembly as cells enter mitosis in each new cell cycle.

Results

Cell Cycle Regulation of CENPs -T and -W

To investigate whether the CENP-T/W complex plays a templating role in propagating centromere identity, we first examined their regulation during the HeLa cell cycle. The relative abundance of CENP-T (NP_079558.3) and -W (NP_001012525.1) transcripts and protein were examined in synchronized populations of HeLa cells [Figure 1]. qPCR analysis of transcripts revealed no periodicity in the expression of CENP-T or CENP-W, while CENP-A exhibited the previously reported upregulation in G2 (Figure 1A) [45]. At the protein level, CENP-T and -W exhibited cyclic behaviour, exhibiting maximal abundance in S-phase ~4-fold greater than the minimum observed in late G2 and M (Figure 1B). The relative abundance of each protein at centromeres was estimated using a tagged transgene, because of the absence of monospecific antibodies suitable for immunocytochemistry. Cell lines constitutively expressing CLIP-tagged constructs of CENP-T or -W and exhibiting normal growth kinetics were prepared (Figure S1A). In addition to normal targeting and cell proliferation, similar constructs tagged with fluorescent proteins formed heterodimeric complexes within the CCAN in human cells, assayed by FRET (A. Hofmeister and S. Diekmann, unpublished observations) and fully replaced endogenous gene products in chicken cells [32], suggesting normal protein function. Asynchronous cultures expressing CLIP-tagged proteins at steady state were labelled in vivo with CLIP-505, fixed and processed for immunodetection with cell cycle markers PCNA and phospho-histone H3 and with a CENP-A monoclonal antibody (Figure S1B). The abundance of CLIP-tagged CENP-W at centromeres was determined by quantitative microscopy and the population classified according to cell cycle stage (Figure 1C). CENP-W was seen to increase at centromeres in correlation with progression through S-phase, approximately doubling in concentration at centromeres in late S-phase and slightly increasing beyond this in G2 cells. CENP-T showed a similar pattern of accumulation (unpublished data). Taken together, these results show that CENPs -T and -W are constitutive centromere proteins whose abundance at centromeres correlates with the state of replication of the nucleosomes.
A. Transcript abundance

B. Time (Hrs)

C. Fluorescence intensity

D. CENP-W CLIP-505

ACN

PCNA
Expression of CENP-W Is Required for Each Mitosis

Cells depleted of CENP-A exhibit a distinct phenotypic response, maintaining normal kinetochore function for two to three cell cycles until a critical threshold of about 10% of normal levels are reached and mitosis fails [33]. This behavior is similar to early results obtained by microinjection of antibodies to CENP-C [46], showing that cells can accommodate the loss of activity of certain CENPs by making smaller yet functional kinetochores. To determine whether depletion of CENPs -T or -W results in a similar delay in the onset of mitotic phenotype, RNAi was performed in HeLa cells using immunofluorescence and live cell microscopy to assay effects of depletion (Figure 2). Depletion of CENP-W for 48 h resulted in severe disruption of mitosis comparable to that reported by conditional depletion in chicken cells [32]. Spindles were frequently multipolar (Figure 2A) and cells exhibited an extended prometaphase with numerous misaligned chromosomes, mono-oriented chromosomes, failure of congression, and pronounced spindle rolling phenotype (Videos S1–S4). Depletion of CENP-T using pooled siRNAs yielded relatively subtle effects, resulting in characteristic fusiform spindle morphology (Figure 2A), high frequencies of misaligned chromosomes, and congression defects and mild delays in mitosis. The differences in phenotype observed for the two subunits of the complex is likely related to partial knockdown of the proteins under our experimental conditions (Figure S2). Defective mitosis was quantitatively examined by measuring the delay in anaphase onset in HeLa H2B-GFP cells 1–2 d after transfection, observing populations for 12-h windows using time-lapse microscopy (Figure 2B). By observing cells starting 24 h after initiation of transfection, the consequences of CENP-W loss on the first mitosis following depletion could be examined, revealing significant increase in the number and severity of mitotic defects in the population. HeLa H2B-GFP cells exhibit an average “pre-anaphase” time of 89 ± 38 min. Following RNAi, nearly one-third of CENP-W depleted cells showed a mitotic delay greater than 1 standard deviation above the mean and this delay was more extensive than that seen in untreated populations, with an average excess time of 154 min following CENP-W depletion (Figure 2B, inset). These defects were much more severe 48 h following transfection. These results indicate that deposition of CENP-W is required in each cell cycle for a robust mitosis. In contrast to the induced mitotic defects, depletion of CENP-T or -W did not disrupt assembly of CENP-A in cells cotransfected with siRNA and an mCherry-CENP-A plasmid (Figures S3 and S4), indicating that the mitotic defects induced by CENP-W depletion appear independently of the CENP-A loading pathway. These results suggest that, within the time frame of one cell cycle, the CENP-T/W complex plays a role primarily in kinetochore function rather than in locus maintenance.

CENPs -T and -W Are Not Stably Inherited

A different perspective on the role of the CENP-T/W complex in locus maintenance was gained by examining multigenerational stability. A pulse-chase experiment was performed using cells expressing CLIP-tagged CENPs -T or -W in conjunction with SNAP-tagged CENP-A, synchronizing cells with a double thymidine block and labelling with a fluorescent ligand at the time of release. Cells were harvested at 24-h intervals and the abundance of protein at centromeres was determined by quantitative fluorescence microscopy (Figure 2C and 2D). CENP-A showed a diminution of signal of approximately 50% per generation over two subsequent cell cycles, while CENP-T and -W CLIP signals were reduced to background within 24 h. Labelling was also performed 6 h after release, in S-phase, and cells were examined after 24 h with similar loss of the pulse population of molecules (unpublished data). CENPs -T and -W thus behave as exchangeable components of centromeric chromatin. We conclude that, unlike CENP-A, this histone H3–associated CENP-T/W histone-fold domain complex is not situated to function as a stably bound, physically heritable marker of centromere identity.

CENPs -T and -W Assemble prior to Mitosis through a Dynamic Exchange Mechanism

In order to examine the time at which the CENP-T/W complex assembles in the cell cycle, a conditional labelling strategy was employed, similar to that used to pinpoint CENP-A assembly timing in HeLa cells (Figure 3A) [22]. Cells expressing CLIP-tagged CENPs -T or -W were synchronized and reactive CLIP proteins were blocked prior to release into the cell cycle. After 6.5 h, newly synthesized CLIP-tagged proteins were labelled and cells were collected at 2-h intervals. Cells were conditioned for tubulin to stage cells as they progressed through mitosis and into the subsequent G1. Cells undergoing mitosis clearly exhibited newly assembled CENP-T and -W at centromeres (Figure 3B), while CENP-A assembly was not detected until telophase/G1 (Figure S5). Thus, unlike CENP-A, newly synthesised CENP-T and -W assemble in the cell cycle in which they are made, prior to the execution of mitosis.

To resolve when CENP-T and CENP-W are assembled within the proximal cell cycle, the CLIP-quench-chase-pulse experiment was repeated and the cells were counterstained with centromere (CENP-A) and cell cycle markers (PCNA and phospho-histone H3); Figure 3C). Cells were scored for CENP-T/W assembly by inspection and also categorized with respect to PCNA and phospho-histone H3 staining, allowing cells to be classified as S phase (PCNA-positive), G2 and M (phospho-histone H3-positive), or G1 (negative for either PCNA or H3P). CLIP-505 signal was detected at baseline levels in cells that were in very early stages of S-phase, identified by fine punctate PCNA staining. However, cells which had progressed further through S-phase displayed robust centromere associated CLIP-505 signal. Quantification of fluorescence intensity demonstrated an increase in centromere associated CLIP-505 signal correlated to progression through S-phase (Figure 3D).

Figure 1. Analysis of CENP-T and -W in the cell cycle. (A) HeLa cells were fractionated across the cell cycle using a double thymidine protocol. The relative abundance of CENP-T (red) and -W (blue) transcripts were measured by qPCR. CENP-A (dashed grey) is shown as a reference. No significant periodic RNA accumulation was observed. (B) Protein obtained from cell cycle fractions was examined by Western blot for CENP-T and CENP-W relative to tubulin (loading control), cyclin B, and phospho-histone H3 (Ser10). (C) The relative abundance of centromere-associated CENP-W was estimated using a cell line constitutively expressing a CLIP-tagged fusion protein. CENP-W-CLIP was labelled with CLIP-505 at steady state and fluorescence intensity quantified. Cells were counterstained for CENP-A to define centromeres and for PCNA and phospho-histone H3 to resolve the cell cycle stage of individual cells (see Figure S2). Cells were scored as S-phase (PCNA-positive), G2 and M (phospho-histone H3-positive), or G1 (negative for either PCNA or H3P). Early and late S-phase designations were made on the basis of PNCA distribution. (D) An example of cell staining showing CLIP-505-CENP-W assembly in a pair of cells in S-phase and undetected in a pair of G2 cells.

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Switching Centromere Chromatin for Mitosis
Switching Centromere Chromatin for Mitosis

A.

CENP-W
MT
ACA
DAPI

CENP-T

untreated

B.

Prophase-anaphase time (min)

rank order

siRNA-W 24h
siRNA-W 48h
siRNA Non Targeting
Untreated

% delayed
delay (min)
siRNA control 13% 34
CENP-W 24 hr 29% 154
CENP-W 48 hr 69% 236

C.

Double Thymidine synchronisation
Release at G1 / S boundary
PULSE with CLIP-80S

24 hr chase to subsequent G1/S boundary
Immunofluorescence & Quantitation of signal

D.

Signal remaining after 24 hr

47.5%
0.0%
-0.2%
The CENP-T/W complex is required in each mitosis and does not exhibit persistent binding to centromeres. (A) CENPs -T and -W were depleted by RNAi in HeLa cells, resulting in defects in spindle assembly assayed by immunofluorescence for tubulin (green) and centromeres (red) with DNA stained with DAPI (blue). GFP-W depleted cells exhibited a high frequency of multipolar spindles. Depletion of CENP-T was less severe resulting in characteristic fusiform spindle structures. (B) CENP-W is required for robust mitosis in each cell cycle. Mitotic kinetics were assayed using histone H2B-GFP HeLa cells treated with CENP-W siRNA for 48 h. (C) The CENP-T/W complex assembles to a fixed stoichiometry along with new histone H3 nucleosomes. CENPs -A and -I exhibit a stable binding kinetic. (unpublished data), demonstrating that the CENP-T/W complex assembles through a dynamic exchange mechanism that is restricted primarily to late S-phase and G2.

Discussion

Investigation of the assembly and inheritance of CENPs -T and -W reveals a dynamic pathway for assembly late in the cell cycle that is associated with rapid exchange of the proteins, such that they do not exhibit multigenerational persistence. As a group, centromere protein assembly occurs through several mechanistically distinct processes distributed throughout the cell cycle. The CENP-A assembly pathway itself is distributed from mid S-phase, when replication of CENP-A associated DNA occurs, through late G1 of the subsequent cell cycle, when RSF-dependent and MgcRacGAP-dependent mechanisms stabilize newly deposited CENP-A [21–26,47,48]. In vivo analysis of GCAN component assembly using FRAP has revealed distinct classes of protein, on the basis of the timing and mechanism, either stoichiometric or dynamic exchange, of assembly [47]. In this study, all proteins examined were stably bound in mitosis with CENPs -A and -I exhibiting stable binding throughout the cell cycle, showing assembly-coupled fluorescence recovery only during telophase/G1 and S-phase, respectively. CENPs -B, -C, and -H exhibited dynamic exchange throughout much of the cell cycle, becoming stably bound only in G2 (CENP-B) or S-phase (CENPs -C and -H). CENPs -T and -W form a novel class that is nonexchangeable during G1, exhibiting assembly-coupled dynamic exchange during S-phase and G2.

The CENP-T/W complex has been shown to interact stably with histone H3 nucleosomes [32]. Their dynamic behaviour revealed by in vivo measurements, as well as differences in their kinetics of assembly, are unexpected for stable chromatin components. The biochemically purified complex could represent a stably assembled population, perhaps comparable to that observed in G1. The onset of recovery of fluorescence in S-phase, which occurs at all centromeres simultaneously, is suggestive of an active switch rather than a passive response to DNA replication at centromeres, suggesting that a biochemically purified complex...
could be stabilized by dissociation from factors that may promote the exchange reactions observed in vivo. The kinetic differences observed for CENP-T and -W exchange in our FRAP studies suggest mechanistic features of their assembly reaction(s) that bear further investigation with refined in vivo analyses.

Although the precise molecular organization of the histone H3-CENP-T/W nucleosome population is not known, it is reasonable to assume that they are interspersed closely with CENP-A nucleosomes, on the basis of known protein-protein interactions [30,31] and measurement of CENP-A oligonucleosome domain size (estimated at four to six contiguous nucleosomes, M. Glynn and K.F. Sullivan, unpublished data). Analysis at the single molecule level in stretched chromatin fibers supports a very close interspersion [48]. In human cells, DNA replication in this compartment occurs in the absence of new CENP-A, resulting in replicative dilution of parental CENP-A nucleosomes [49]. It is thought that histone H3 nucleosomes assemble in their place [12,24]. We propose that CENPs -T and -W, assemble onto histone H3 nucleosomes within this compartment. The functional consequence of these assembly events would be an expansion of the histone H3/CENP-T/W compartment within postreplicative centromeric chromatin. The dynamic behaviour of protein within this compartment kinetically parallels the active establishment of the kinetochore complex, which spans the period from G2 to early mitosis in human cells [50,51]. Taken together with the known involvement of the CCAN with kinetochore formation, we suggest that assembly of the CENP-T/W complex plays a functional role in kinetochore formation following DNA replication.

The immediate requirement for CENP-W for successful execution of mitosis is consistent with the CENP-T/W complex playing an active role in kinetochore assembly in G2 and contrasts starkly with the ability of cells to accommodate loss of CENP-A over multiple generations without defect [33]. The complementary kinetics of their assembly leads to a view in which CENP-A serves a role as a placeholder, diminishing in proportion as a more direct kinetochore chromatin foundation is built up prior to mitosis. CENP-A has a distinct role as a carrier of centromere identity over multiple generations. Its replenishment cycle appears to be complete at the onset of S-phase [24–26]. We suggest that this “fully loaded” CENP-A state corresponds to replication-competent centromeric chromatin. Replication through CENP-A chromatin would initiate a switch of the centromere to a kinetochore-competent configuration, accompanied by assembly of the CENP-T/W complex onto histone H3 nucleosomes, providing an expanded platform for assembly of additional CCAN components. The lack of generational persistence of the CENP-T/W histone fold complex suggests that it is not a stably associated molecular mark for centromere identity at the chromatin level, nor does it appear to play a direct role in CENP-A assembly.

The broadly used definitions of the centromere as a genetic locus, e.g. DNA, and the kinetochore as the facultative, proteinaceous structure on the primary constriction that executes mitotic function have lost distinction with the demonstration that a chromatin protein complex carries the genetic function of the locus. The different behaviour of the CENP-A and CENP-T/W chromatin compartments suggest a degree of segregation of genetic (centromere) function and mitotic (kinetochore) function within biochemically specialized chromatin microdomains of the centromere. Investigation of the roles of individual CCAN components in the maintenance of centromere identity, establishment of kinetochore function, and the integration of these two processes may provide important paradigms for understanding epigenetic chromatin-based inheritance.

**Methods**

**Cell Culture and Transfection**

Cells were cultured as previously described [52]. siGENOME SMARTpool siRNAs (Dharmacon) M-014577-01 and M-032901-01 were used to deplete CENP-T and CENP-W respectively. siRNA was transfected into cells using DharmaFECT 1 (Dharmacon). Cells were transfected with plasmid DNA with Lipofecta-

**Constructs**

pCLIPm (NEB) was adapted for use with the Gateway system (Invitrogen) by linearising the plasmid in the MCS by restriction digest with EcoRV. Gateway adaptation reading frame cassette RFB (Invitrogen) was ligated into the plasmid to generate a pCLIPm for C-terminal CLIP tagging (GW-pCLIPm). AnB flanked PCR products were generated from cDNA for CENP-T and CENP-W and recombined with pDouc-Zeo (Invitrogen) to generate entry vectors, which were recombined with GW-pCLIPm to generate the CENP-T-CLIP and CENP-W-CLIP constructs.

**Cell Lines**

Cells stably expressing the CENP-A-SNAP fusion protein were a gift from L.E. Jansen. The CENP-A-SNAP cell line was transfected with the CENP-T-CLIP and CENP-W-CLIP constructs. Cells stably expressing the fusion proteins were selected by G418 (600 μg/ml; Calbiochem). The resulting monoclonal lines were expanded and examined by fluorescence microscopy after CLIP-505 labelling. CENP-T-CLIP 11 and CENP-W-CLIP 4 were used for all experiments in this paper. These lines exhibited population doubling times and cell cycle distributions indistinguishable from the parental line.

**CLIP Quench and Pulse Labelling**

CLIP tag activity in cells was quenched by addition of 20 μM O6-BC (BC-block; NEB) in complete growth medium for 30 min at 37°C. SNAP or CLIP-tag proteins were pulse labelled with 2 μM CLIP-505/CLIP-Tmr Star/SNAP-Tmr Star (NEB) in complete growth medium supplemented with 1% BSA for 20 min at 37°C. After quenching or pulse labelling, cells were
were then subtracted from mean signal. Background values were deconvolved and maximum intensity projected using SoftWoRx (Applied Precision). For quantification, unscaled DeltaVif were deconvolved and maximum intensity projected using SoftWoRx (Applied Precision). Cell Synchronization

HeLa cells were treated with 5 mM thymidine in complete DMEM for 16 h, washed twice in PBS, once in complete DMEM, and released in complete DMEM 9 h followed by addition of thymidine to a final concentration of 5 mM for 16 h, after which cells were released again into complete DMEM.

Microscopy

Cells were grown and CLIP labelled on glass coverslips followed by fixation in MeOH or 4% PFA and processed for immunofluorescence. ACA (1:500), Anti-CENP-A (a gift from K. Yoda, Nagoya University, Nagoya, Japan) was used at a dilution of 1:200, human anti-PCNA serum was used at 1:100, anti-X-tubulin (Sigma) was used at 1:500, and anti-phospho histone H3 (Abcam) was used at 1:300.

Donkey fluorescently conjugated secondary antibodies (anti-mouse-FITC (1:50) anti-mouse Cy5 (1:200), anti-rabbit TRITC (1:100), and anti-human AMCA (1:100) were obtained from Jackson Immunoresearch Laboratories. Samples were mounted in SloFADE (Invitrogen).

Supporting Information

Immunofluorescence

Cells were separated by SDS-PAGE, transferred to PVDF membranes (Millipore), and processed for immunodetection as described previously [49]. Antibodies: anti-CENP-T (Bethyl), anti-CENP-W (C6ORF173 antibody Abcam, ab75827), anti-tubulin monoclonal (DN1A Sigma), anti-H3P Ser10 (Millipore), cyclin-B (Upstate).

Figure S1 Analysis of CENP-A assembly following CENP-W depletion. (A) Experimental schematic describes the approach to assay assembly of CENP-A following depletion of CENP-W by siRNA. HeLa cells expressing H2B-GFP were cotransfected with mCherry-CENP-A and siRNA directed against CENP-W. After 48 h, cells were fixed for immunofluorescence or treated with an Aurora B inhibitor (ZM44739, JS Research Chemicals) to relive mitotic delay associated with CENP-W depletion. Cells were examined for assembly of mCherry-CENP-A to centromeres. (B) Immunofluorescence in H2B-GFP HeLa cells using centromere marker (ACA) and tubulin following depletion of CENP-W. mCherry-CENP-A is observed at centromeres in early G1 cells displaying a tubulin staining of the midbody, while some transfected metaphase cells, indicated by presence of mCherry signal, with uncongressed chromosomes, have not assembled CENP-A at centromeres diffuse mCherry signal. (C) Cells were also treated with an Aurora B inhibitor, following 48 h CENP-W depletion. Cells judged to have entered G1 as a result of Aurora B inhibition exhibited abnormal nuclear morphology and centromeres that in most cases had failed to segregate. Transfected cells positive for mCherry-CENP-A had assembled CENP-A at centromeres.

Figure S2 Quantitative reverse transcript (RT)-PCR was used to measure the levels of depletion of both CENP-T and CENP-W transcripts following a 48-h siRNA treatment. CENP-W was depleted to approximately 48% while CENP-T transcripts were reduced to approximately 36%. Corresponding Western blots shown below demonstrate comparable levels of protein depletion.

Figure S3 Analysis of CENP-A assembly following CENP-W depletion. Cells were synchronized by reincubation in complete DMEM, or fixation. Following washes cells were reincubated in complete medium to allow excess labelling compound to diffuse from cells. After 30 min, cells were washed again twice in PBS followed either by reincubation in complete DMEM, or fixation.
subsequent G1, as previously reported. Insets detail SNAP-505 and ACA staining in interphase and mitotic cells.

**Figure S6 FRAP of GFP derivatives of CENP-W only occurs during late S-phase and continues during G2, indicating loading takes place during this time.** Experiment analogous to that shown for CENP-T in Figure 4. (EPS)

**Video S1 CENP-W knockdown induces profound mitotic delays and induces chromosome/spindle rolling in cells.** HeLa-H2B-GFP cells were transfected with pooled siRNAs against CENP-W and imaged for 12 h beginning 48 h after transfection, with frames collected every 5 min. This sequence shows a projected image superimposed on a transmitted light image of cells. Two rolling events collected every 5 min. This sequence shows a projected image superimposed on a transmitted light image of cells. Two rolling events are visible, one of which is clear that the chromosomes are moving collectively, characterized by the metaphase plate rotating 90° continuously motion through almost 180° superimposed on a transmitted light image of cells. Two rolling events are visible, one of which is clear that the chromosomes are moving collectively, characterized by the metaphase plate rotating 90° continuously motion through almost 180°.

**Video S2 4-Dimensional imaging of rolling spindles: projection.** HeLa-H2B-GFP cells transfected with pooled siRNAs against CENP-W were imaged by 3-dimensional time-lapse microscopy beginning 48 h after transfection, with frames collected every 5 min. This sequence shows a projected image superimposed on a transmitted light image of cells. Two rolling events are visible, one of which is clear that the chromosomes are moving collectively, characterized by the metaphase plate rotating 90° continuously motion through almost 180°.

**Video S3 4-Dimensional imaging of rolling spindles: reconstruction.** HeLa-H2B-GFP cells transfected with pooled siRNAs against CENP-W were imaged by 3-dimensional time-lapse microscopy beginning 48 h after transfection, with frames collected every 5 min. In this sequence, the field is rocked to reveal the 3-dimensional structure of the chromosomes on the spindle. It is clear that the chromosomes are moving collectively, characterized by the metaphase plate rotating 90° continuously motion through almost 180°.

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**Author Contributions**

The author(s) have made the following declarations about their contributions: Conceived and designed the experiments: LP CaV SD KFS. Performed the experiments: LP CaV AK NQ SD KFS. Analyzed the data: LP CaV AK NQ DH CH KFS. Contributed reagents/materials/analysis tools: LP CaV AK NQ SD KFS. Wrote the paper: LP SD KFS.