Molecular control of kinetochore-microtubule dynamics and chromosome oscillations

Ana C. Amaro, Catarina P. Samora, René Holtackers, Enxiu Wang, Isabel J. Kingston, Maria Alonso, Michael Lampson, Andrew D. McAinsh and Patrick Meraldi

Chromosome segregation in metazoans requires the alignment of sister kinetochores on the metaphase plate. During chromosome alignment, bioriented kinetochores move chromosomes by regulating the plus-end dynamics of the attached microtubules. The bundles of kinetochore-bound microtubules alternate between growth and shrinkage, leading to regular oscillations along the spindle axis. However, the molecular mechanisms that coordinate microtubule plus-end dynamics remain unknown. Here we show that centromere protein (CENP)-H, a subunit of the CENP-A nucleosome-associated and CENP-A distal complexes (CENP-A NAC/CAD), is essential for this coordination, because kinetochores lacking CENP-H establish bioriented attachments but fail to generate regular oscillations, as a result of an uncontrolled rate of microtubule plus-end turnover. These alterations lead to rapid erratic movements that disrupt metaphase plate organization. We also show that the abundance of the CENP-A NAC/CAD subunits CENP-H and CENP-I dynamically change on individual sister kinetochores in vivo, because they preferentially bind the sister kinetochore attached to growing microtubules, and that one other subunit, CENP-Q, binds microtubules in vitro. We therefore propose that CENP-A NAC/CAD is a direct regulator of kinetochore-microtubule dynamics, which physically links centromeric DNA to microtubule plus ends.

Chromosome alignment is a multistep process in which kinetochores establish stable bioriented attachments to the plus end of spindle microtubules (MTs). The main components required for bioriented attachments are the KMN (Knl1–Mis12–Ndc80) kinetochore complex, which binds and stabilizes end-on-attached MTs, and error-correction mechanisms based on the protein kinase Aurora B, which detaches improper kinetochore–MT (kMT) attachments by phosphorylating the KMN complex. In mammalian cells, bioriented sister–kinetochore pairs on the metaphase plate undergo regular oscillations along the spindle axis driven by coupled MT depolymerization at the leading kinetochore and MT polymerization at the trailing kinetochore. Importantly, 25–30 MT plus ends bind each kinetochore to form MT bundles, known as kinetochore fibres (k fibres). Although k fibres are embedded into kinetochores, the plus ends of the individual MTs remain dynamic, with a rate of tubulin turnover 20-fold slower than the plus ends of free spindle-MTs. The key to understanding how kinetochores generate directional movement and align chromosomes is to identify the proteins and molecular mechanisms that coordinate the dynamics of individual MT plus ends within k fibres.

Several MT-associated proteins (MAPs) accumulate onto end-on attached kinetochores and regulate kMT dynamics. These include the kinesins Kif18a and mitotic centromere-associated kinesin (MCAK), which possess MT-depolymerizing activity, and members of the CLIP-associated protein (CLASP) and EB protein families. Kif18a and MCAK modulate the speed of kinetochore oscillations but do not coordinate MT plus-end dynamics, because their depletion does not abrogate chromosome oscillations. Hec1—a component of the MT-binding NDC80 complex—or the introduction of a Hec1 mutant that cannot be phosphorylated by Aurora B suppresses kMT tubulin turnover and disrupts chromosome alignment in Ptk1 cells. Finally, CLASPs have been implicated in the regulation of kMT plus-end dynamics and the generation of poleward MT flux in various systems. However, because these MAPs also associate with the plus ends of free spindle-MTs and because the plus ends of free MTs and kinetochore MTs have different tubulin turnovers, one has to assume that kinetochore-specific factors must coordinate kMT plus-end dynamics.

Consistent with this hypothesis, microinjection of antibodies against Hec1—a component of the MT-binding NDC80 complex—or the introduction of a Hec1 mutant that cannot be phosphorylated by Aurora B disrupts chromosome alignment in Ptk1 cells. However, this result reflects a gain of function of Hec1, because loss of Hec1 disrupts MT attachments. Other potential candidates are the 15 proteins that associate with centromere-specific, CENP-A-containing nucleosomes (CENP-H/I/K/L/M/N/O/P/Q/R/S/T/U/W/X), and form a protein network termed CENP-A NAC/CAD, which physically links centromeric DNA to microtubule plus ends.
ICEN or CCAN complex. Loss of CENP-H, CENP-I, CENP-K or CENP-N, which disrupts the kinetochore localization of all of these proteins except CENP-T/W, does not affect the ability of kinetochores to bind MT plus ends, but instead impairs chromosome congression. Moreover, depletion of CENP-O (also known as Mcm21R) disrupts the kinetochore localization of CENP-U/P/Q/R and destabilizes K fibres.

Figure 1 Loss of CENP-H abolishes sister-kinetochore oscillations and disrupts metaphase plate alignment. (a) Live-cell stills of single kinetochore pair oscillations in wild-type (WT) or CENP-H-depleted EGFP–CENP-A HeLa cells. Scale bar, 2 µm. (b) Autocorrelation function of translational sister-kinetochore movements along the spindle axis (kinetochore oscillation) of siControl (black line), siCENP-H (red line) or fixed cells (green line). The autocorrelation function was calculated by combining all aligned sister-kinetochore pairs for each condition (Supplementary Information, Table S2). The first negative lobe in control-depleted cells (black arrow) indicates the half-period of the mean oscillation period, and its depth indicates the oscillation regularity. The second positive lobe (grey arrow) indicates the full period. Note that a random motion always produces a negative value at the first lag. (c–e) Histograms of the mean interval time between directional switches of the sister-kinetochore pairs along the spindle axis of siControl (c), siCENP-H (d) or fixed cells (e). Means ± s.d. values: 22.06 ± 15.15 s (c), 15.73 ± 12.26 s (d) and 23.46 ± 26.47 s (e). (f) Average sister-kinetochore pair velocity along the spindle axis of siControl, siCENP-H and fixed cells. Error bars represent s.d. for n = 3 independent experiments. (g) Width of the metaphase plate of siControl, siCENP-H and fixed cells. Error bars represent s.d. for n = 3 independent experiments. (h) Representative images of control (top) or CENP-H-depleted (bottom) cells arrested in metaphase for 1 h with the proteasome inhibitor MG132 and stained with 4,6-diamidino-2-phenylindole (DAPI; for DNA; blue), anti-α-tubulin (MTs; green) and CREST antisera (kinetochores; red). Scale bar, 10 µm. (i) Percentage of cells with one, two, three or more than three uncongressed chromosomes after a 1-h treatment with MG132 calculated from images such as those shown in h. Chromosomes in metaphase cells were counted as unaligned if they were located outside the central 30% of the mitotic spindle. Error bars represent s.e.m. for n = 3 independent experiments with 20 cells each.
thus potentially linking CENP-A NAC/CAD to k-fibre regulation. We therefore investigated whether and how CENP-A NAC/CAD subunits contribute to the regulation of kMT plus-end dynamics.

RESULTS

Loss of CENP-H disrupts sister-kinetochore oscillations and metaphase plate alignment

To investigate whether CENP-A NAC/CAD is involved in the control of kMT dynamics and kinetochore oscillations, we depleted the CENP-H subunit, which is required for the loading of CENP-H/I/K/L control of kMT dynamics and kinetochore oscillations, we depleted the metaphase plate alignment loss of CENP-H disrupts sister-kinetochore oscillations and therefore investigated whether and how CENP-A NAC/CAD subunits thus potentially linking CENP-A NAC/CAD to k-fibre regulation. We

Figure 2 Loss of CENP-H abrogates MT flux and abolishes control of kMT turnover. (a) Successive frames every 30 seconds before and after photoactivation of stable photoactivatable GFP (PAGFP)-α-tubulin/H2B-mRFP HeLa cells treated with siControl, siCENP-H or siMCAK + siKif2a RNAs. Photoactivatable GFP-α-tubulin fluorescence was activated in a circular region near the chromosome mass in metaphase cells (detected by the H2B-mRFP signal). An H2B-mRFP (DNA) frame is shown for the first time point of the live-cell movie after activation. (b) Quantification of poleward MT flux rates in cells treated with siControl (black bars), siCENP-H (red bars) or siMCAK + siKif2a (green bars) RNAs. n = 20 cells each. (c, d) Quantification of fluorescence intensity decay of the activated regions over time in siControl-treated (black; c, d), siCENP-H-treated (red; c) or siMCAK + siKif2a-treated cells (green; d). The lines through the data points were fitted to a double-exponential equation of the type \( f(t) = P_1 \exp(-k_1 t) + P_2 \exp(-k_2 t), \) corresponding to described previously slow and fast MT populations7,19. Analysis of the siCENP-H fluorescence loss indicated that the data fitted to single-exponential curve (R² = 0.99). Indicated are the corresponding half-lives of the fast and slow MT populations. Green arrowheads in a mark the initial position of the photoactivated spot; red arrowheads show the final position of the activated spot. Scale bar, 10 µm. Error bars represent s.d.

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with 1.18 µm in control-depleted cells; Fig. 1g). These metaphase plate organization defects are the likely reason for the chromosome alignment defects in CENP-H-depleted cells (Fig. 1h, i)\textsuperscript{27,31}. We conclude that CENP-H loss abrogates regular chromosome oscillations and disorganizes metaphase plate alignment, suggesting a crucial role in the regulation of kMT plus-end dynamics.

Loss of CENP-H hyperstabilizes k fibres and elevates plus-end tubulin turnover

To test how CENP-H depletion affects kMT plus-end dynamics, we established a stable HeLa cell line expressing histone H2B conjugated with monomeric red fluorescent protein (mRFP) to identify metaphase cells, and photoactivatable GFP–α-tubulin to measure tubulin turnover at MT plus ends. We generated fluorescent marks on the metaphase plate and monitored two parameters: first, the movement of the marks towards the poles as a measure of poleward MT flux, which reflects MT treadmilling, and second, the time-dependent decrease in fluorescence intensity of the activated marks, which provides a readout of tubulin turnover at MT plus ends (expressed as the half-life, \( t_{1/2} \)). In control-depleted cells, the activated marks moved towards the spindle pole with a mean rate of 0.9 ± 0.3 s.d. µm min\(^{-1} \) (Fig. 2a, b; Supplementary Information, Video S1). The rate of plus-end tubulin turnover was stable kMTs in cold-treated cells. The bar graph indicates the percentage of cells with all kMTs intact (Cat1), lacking few kMTs (Cat2), possessing few short kMTs left (Cat3) or possessing no kMTs (Cat4). (d) Representative images of cells treated with siControl, siCENP-H or siControl + Taxol RNAs and stained with anti-acetylated-α-tubulin (stable MTs; green), anti-α-tubulin antibodies (MTs; red) and CREST antisera (kinetochores; blue). \( n = 50 \) cells. Scale bar, 10 µm. (e) Immunofluorescence quantification of the ratio of acetylated-α-tubulin to α-tubulin in mitotic spindles of cells after treatment with siControl, siCENP-H or siControl + Taxol from images such as those shown in d. \( n = 70 \) cells. Error bars represent s.e.m. for \( n = 3 \) independent experiments.
Figure 4 CENP-A NAC/CAD regulates kMT dynamics independently of Aurora B/KMN. (a) Graphs showing the Aurora B FRET-based sensor emission ratio of YFP/CFP for mock-treated (siControl), ZM1 (Aurora B inhibitor)-treated and siCENP-H-treated cells. An increase in emission ratio indicates dephosphorylation (lower Aurora B activity). Error bars represent s.e.m. for n = 3 independent experiments. (b) Representative images of siControl or siCENP-H-treated cells fixed after FRET analysis and stained with CENP-H antisera. (c–f) Representative images of siControl or siCENP-H RNA-treated cells stained with CREST or CENP-A (red) and Hec1 (c), Nnf1R (d), CENP-E (e) or MCAK (f) antisera (green). Insets show higher-magnification views of a single kinetochore. (g) Representative images of HeLa cells stably expressing EGFP–CLASP1 treated with siControl or siCENP-H RNAs and either no drug, 1 μM Taxol or 1 μg ml⁻¹ nocodazole (noc.). Cells were stained with CREST antisera (to mark kinetochores; red). Insets show higher-magnification views of a single kinetochore. (h) Quantification of the EGFP–CLASP1 signal on kinetochores normalized with the signal at the spindle poles (see Supplementary Information, Fig. S3, for methodological details for indicated treatments); n > 25 kinetochores for each treatment. Error bars represent s.e.m. for n = 3 independent experiments. (i) Representative images of HeLa cells transiently expressing EGFP–CLASP2 treated with siControl or siCENP-H and stained with CREST antisera (to mark kinetochores; red). Insets show higher-magnification views of a single kinetochore. (j) Representative images of siControl or siCENP-H RNA-treated cells stained with CREST (to mark kinetochores; red) and EB1 antisera (green). (k) Immunofluorescence quantification of EB1 levels on the metaphase plate region after treatment with siControl or siCENP-H RNAs from images such as those shown in j, n = 25 cells. 10 μm.
biphasic, reflecting the two previously reported MT populations: a fast population of free spindle MTs ($t_{1/2} = 8.9 \pm 0.9$ s, 75% of all MTs), and a second population with low turnover, which represents k fibres ($t_{1/2} = 209.1 \pm 21.1$ s, 25% of all MTs; Fig. 2a, c). In contrast, in CENP-H-depleted cells, MT flux was abrogated in 75% of the cells (Fig. 2a, b; Supplementary Information, Video S2). Moreover, the tubulin turnover at plus ends was very rapid (Fig. 2a, c) and the data set could be fitted to a single exponential with a half-life similar to that of plus ends in free spindle MTs ($t_{1/2} = 10.1 \pm 0.7$ s; Fig. 2c). As reported previously, simultaneous depletion of the MT depolymerases Kif2a and MCAK did not affect plus-end tubulin turnover, even though MT flux was severely decreased, indicating that the high turnover rate of plus ends in CENP-H-depleted cells was not due to a decreased poleward MT flux (Fig. 2a, b, d)

The presence of a single population of plus ends, showing a high turnover rate, could be explained either, first, if CENP-H-depleted cells had a minimal number of MTs within k fibres, which could explain the difficulty of coordinating directional movements, or second, if CENP-H-depleted kinetochores could not reduce the turnover of plus ends in k fibres. To differentiate between those possibilities, we measured whether bioriented sister kinetochores on the metaphase plate have normal distances between kinetochores, because a decrease in kMT numbers within a k fibre decreases this distance. We found that CENP-H-depleted and control-depleted cells had the same distances between kinetochores and that this distance was decreased to the same extent when k fibres were disrupted by the MT-depolymerizing drug nocodazole (Fig. 3a). To reveal k fibres we used a cold-stable assay. A 10-min treatment with ice-cold medium depolymerized all non-kMTs in control-depleted cells, whereas longer treatments of up to 30 min progressively decreased k fibres (Fig. 3b, c). Cells lacking the Nde80 complex component Nuf2R had no k fibres after 10 min of cold treatment, which is consistent with previous reports (Fig. 3b, c). In contrast, in CENP-H-depleted cells the k fibre network was as intense as in control-depleted cells. Moreover, these k fibres were more resistant to cold: many long k fibres persisted even after a 30-min cold treatment (Fig. 3b, c). This indicated that CENP-H-depleted cells have k fibres with a normal number of MTs, and implied that their MT lattice is hyperstable, whereas their plus ends have a high tubulin turnover. To analyse the turnover of the spindle-MT lattice, we measured the immunofluorescence ratio of acetylated α-tubulin to normal α-tubulin to differentiate between kinetochores bound to growing or shrinking MTs, providing a readout of MT turnover. Both treatment with Taxol and depletion of CENP-H increased the levels of tubulin acetylation by 55% in comparison with control-depleted cells (Fig. 3d, e). We conclude that, in CENP-H-depleted cells, the k fibre lattice is hyperstable, whereas the plus ends are rapidly turned over.

**CENP-A NAC/CAD controls plus-end dynamics independently of Aurora B and binds differentially to kinetochores bound to growing or shrinking k fibres**

How does CENP-H control kMT plus-end turnover at the molecular level? One possibility is that the Aurora B/KMN network could be affected by a loss of CENP-H. However, using a fluorescence resonance energy transfer (FRET)-based reporter assay, we found that CENP-H depletion did not influence the kinase activity of Aurora B (Fig. 4a, b). Moreover, multiple components of the KMN network, MCAK and CENP-E, all downstream targets of Aurora B, remained kinetochore-bound in the absence of CENP-H, indicating that CENP-H depletion affects kMT plus-end dynamics independently of Aurora B and KMN complexes (Fig. 4c–f, but see also refs 27, 37–40). In addition, CENP-H depletion did not impair the recruitment at kinetochores of p150 snoold (dynein complex), CENP-F, the kinesin Kif18a, the spindle checkpoint proteins Bub1 and BubR1, the cohesion factor Sgo1 or Ska1, a component of the MT-binding Ska complex (Supplementary Information, Fig. S2). This indicated that CENP-H depletion does not act as a scaffold for kinetochore-bound MT-regulators.

Another possibility is that CENP-A NAC/CAD provides a scaffold for the recruitment of MAPs to kinetochores. Quantitative immunofluorescence indicated that CENP-H loss abrogated the binding of CLASP1 and CLASP2 to kinetochores and led to a twofold decrease in kinetochore-bound EB1 (Fig. 4g–k). This phenotype was specific, because depletion of the KMN components Nuf2R and Nuf1R (Msi1 complex) did not affect CLASP binding (Supplementary Information, Table S1). However, because blocking kMT plus-end turnover decreases the levels of CLASPs on kinetochores in Ptk1 cells, we treated CENP-H-depleted cells with Taxol or nocodazole to test whether the loss of CLASP on kinetochores reflects modified kMT dynamics. Such treatments restored the levels of kinetochore-bound CLASP1 to 42% and 103%, respectively, of control levels, indicating that CENP-H depletion affects CLASP levels at kinetochores because it changes MT dynamics (Fig. 4g, h).

In the reciprocal experiments, CLASP1 depletion doubled the levels of the CENP-A NAC/CAD components CENP-H and CENP-I on kinetochores but did not affect the levels of the CENP-A NAC/CAD components CENP-O and CENP-P or those of Nuf1R (Fig. 5a, b). Given that CLASPs regulate kMT dynamics, this suggested that the stoichiometry of the CENP-A NAC/CAD network at kinetochores is modulated by changes in kMT dynamics. Consistent with this hypothesis, blocking kMT dynamics in siCLASP1–treated cells with Taxol decreased the abundance of kinetochore-bound CENP-H and CENP-I to normal levels (Fig. 5a, b). We next determined whether CENP-H and CENP-I levels at kinetochores are also sensitive to the state of kMT dynamics in unperturbed cells. Metaphase kinetochore pairs have a leading sister kinetochore bound to depolymerizing kMTs, and a trailing sister kinetochore bound to growing kMTs. If CENP-I and CENP-H protein levels were sensitive to kMT dynamics then one would predict an asymmetry in CENP-I and CENP-H signal intensity between the two sisters. Using immunofluorescence we measured the CENP-I and CENP-H levels of each sister kinetochore and calculated by how much the intensities on individual sister kinetochores differed from the average intensity of the kinetochore pair (Fig. 5c). This revealed a bimodal distribution for both proteins, indicating the existence of two types of sister-kinetochore population, one with CENP-I and CENP-H levels 15% lower than the average, and the other 15% higher than the average (Fig. 5d, h). The asymmetry in CENP-I and CENP-H levels was dependent on MT dynamics, because treatment with Taxol yielded a unimodal distribution (Fig. 5e, i). In contrast, CENP-A, CENP-O and CENP-P had a unimodal distribution peaking at 0 in untreated or Taxol-treated cells, indicating that their levels are unaffected by changes in kMT dynamics (Fig. 5f, g, j–m). This implied that CENP-A NAC/CAD differentiates between kinetochores bound to growing or shrinking MTs, by incorporating more or less CENP-I/H in response to changes in MT dynamics. To test this model, we established stable cell lines expressing...
Figure 5 CENP-H and CENP-I bind differentially to kinetochores attached to growing kinetochore fibres. (a) Representative images of siControl, siCLASP1, siControl + Taxol or siCENP-H + Taxol cells stained with CENP-A antisera (to mark kinetochores; green) and CENP-H antisera (red). Insets show higher-magnification views of a single kinetochore. CENP-A antisera (to mark kinetochores; green) and CENP-H antisera (white). Insets show higher-magnification views of a single kinetochore.

(b) Immunofluorescence quantification of CENP-H, CENP-I, CENP-O, CENP-P and Nnf1R kinetochore levels in cells treated with siControl, siCENP-H, siControl + Taxol or siCENP-H + Taxol RNAs from images such as those shown in a. Error bars represent s.e.m. for n = 3 independent experiments. (c–m) To calculate the asymmetry of the CENP-I (d, e), CENP-A (f, g), CENP-H (h, i), CENP-O (j, k) and CENP-P (l, m) intensities on sister-kinetochore pairs, we determined the intensities of the ‘left’ sister kinetochore (I_l), the ‘right’ sister kinetochore (I_r) and the background value (b) as formulated in c. If one assumes that x is the average signal intensity of one pair, then the left sister kinetochore will have an intensity I_l = x + Δ and the right sister kinetochore will have an intensity I_r = x − Δ (Δ being the relative positive or negative amount by which the two sister kinetochores deviate from the average). By dividing the difference of the background-subtracted intensities over their sum, one obtains Δx/x, which indicates by how much the intensity of the left kinetochore is larger or smaller than the average of the two sisters. We then plotted the distribution of Δx/x in cells treated with (e, g, i, k, m; grey bars) or without (d, f, h, j, l; black bars) Taxol as indicated. A bimodal distribution indicates the existence of two separate sister-kinetochore populations, whereas a unimodal distribution indicates the absence of protein-level asymmetry. Note that CENP-I and CENP-A intensities were measured from the same sister kinetochores (n = 200 kinetochore pairs for CENP-I and CENP-A; n = 100 kinetochore pairs for CENP-H, CENP-O and CENP-P). Scale bars, 10 μm.
either EGFP–CENP-I or EGFP–CENP-O. Using live cell imaging, we
found that the levels of CENP-I on oscillating kinetochore pairs were
asymmetric, because the protein accumulates preferentially at anti-pole-
ward-moving kinetochores attached to growing MTs (30% higher levels;
Fig. 6a, c; Supplementary Information, Video S3). In contrast, the levels
of CENP-O were symmetrical (Fig. 6b, c). We conclude that CENP-I
and CENP-H accumulate on the sister kinetochores bound to growing
k fibres in a manner similar to that of EB1 or Kif18a10,14, suggesting that
CENP-O was symmetrical (Fig. 6b, c). We conclude that CENP-I
and CENP-H accumulate on the sister kinetochores bound to growing
k fibres in a manner similar to that of EB1 or Kif18a10,14, suggesting that
CENP-Q binds the MT lattice and does not preferentially bind MT plus
ends. (Fig. 7f). We deter-
scribed MTs CENP-Q appeared in the pellet fractions in a concentration-dependent
CENP-Q complex can bind MTs. In the absence of Taxol-stabilized MTs,
calculations were performed as in Fig. 5c, but in live cells and defining
that the ‘left’ kinetochore was the sister kinetochore that moved in an
anti-poleward manner and was bound to growing MTs. Error bars represent
Figure 6 CENP-I accumulates preferentially on the sister kinetochore
bound to growing MTs. (a, b) Representative live-cell imaging frames of
single oscillating sister-kinetochore pairs of wild-type EGFP–CENP-I (a) or
EGFP–CENP-O (b) cells. (c) Enrichment of EGFP–CENP-I or EGFP–CENP-O
on anti-poleward-moving sister kinetochores. The quantifications and

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Ska complexes, and second, regulation of kMT plus-end dynamics by CENP-A NAC/CAD (Fig. 8a). Kinetochores lacking CENP-H, and thus lacking all CENP-A NAC/CAD subunits, with the exception of histone H3-binding CENP-T/W subunits, still recruit and stabilize kMTs through attachments to the MT lattice. However, these kMT plus ends undergo fast tubulin turnover, resulting in rapid switches between growth and shrinkage, while leaving the distal lattice region stable (Fig. 8b). The high turnover rate explains the absence of poleward MT flux in 75% of the

Figure 7 The CENP-A NAC/CAD subunit CENP-Q makes direct physical interactions with MTs. (a) SDS–PAGE and Coomassie staining of flow-through (FT), input (I), pellet (P) and elution fractions from Ni²⁺-NTA chromatography. The band corresponding to CENP-Q is indicated on the basis of a mass spectroscopy analysis. (b) Immunoblot of the eluted fraction with anti-CENP-Q antisera. The asterisk-marked band corresponds to degradation products of CENP-Q. WB, western blot. (c) Hydrodynamic analysis of CENP-Q by size-exclusion chromatography. Arrows indicate the elution volumes of standards with known Stokes radii: thyroglobulin (85 Å), ferritin (62 Å), catalase (52 Å) and aldolase (48 Å). Fractions A3–A10 are the void. (d) Fractions from a 5–40% glycerol-gradient analysis of CENP-Q. Arrows indicate the migration of standards with known S values: catalase (11.3 S), aldolase (7.4 S), BSA (4.3 S) and ovalbumin (3.55 S). (e) Hydrodynamic model for the predicted organization of CENP-Q on the basis of the biochemical experiments. (f) MT-pelleting assay performed in BRB80 plus 50 mM NaCl, 1 mM ADP with 1.5 µM Taxol-stabilized pig-brain MTs and purified CENP-Q protein in different concentrations (0.64–3.2 µM) as indicated. Supernatant (S) and pellet (P) fractions are shown. (g) Quantification of binding affinity of CENP-Q for MTs. Quantification was performed using 0.4 µM Taxol-stabilized pig-brain MTs and purified CENP-Q protein (0.64–3.2 µM). Averages of multiple samples are plotted. Error bars indicate s.d. Uncropped images of blots are shown in Supplementary Information, Fig. S6.

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cells: newly incorporated tubulin subunits are rapidly removed from the plus ends, thus preventing MT treadmill (we also observe an increase in MT nucleation at spindle poles, which explains why spindles do not shorten; A.C.A., C.P.S., A.D.M. and P.M., unpublished observation). Our model is consistent with the fact that CENP-H depletion affects kMT plus-end dynamics independently of Aurora B and KMN. We propose that CENP-A NAC/CAD does not act as a scaffold but is a genuine direct regulator of kMT plus ends. Indeed, recent high-resolution imaging of human kinetochore proteins shows that CENP-A NAC/CAD is located in the immediate vicinity of the kMT plus ends\(^4\). Moreover, the Ndc80/Nuf2 and KNL-1 subunits and Ska1/Ska2 subunits are essential for MT attachment, CENP-A NAC/CAD regulates the turnover of kMT plus ends, by controlling dynamic instability. There is a differential stoichiometry of subunits within the CENP-A NAC/CAD complex that is dependent on whether a sister kinetochore is bound to growing or shrinking MTs. The sister kinetochore bound to growing microtubules contains high levels of the CENP-H, CENP-I, CENP-K, CENP-M complex (red), whereas the sister kinetochore bound to shrinking MTs contains low levels of this complex (green). (b) Explanatory model for the lack of correlation between kMT stability and plus-end MT turnover. In control cells, kMT plus ends show low rates of dynamic instability, allowing the continual incorporation of new tubulin heterodimers. In contrast, in the absence of the CENP-A NAC/CAD complex, kinetochores still bind and stabilize kMTs through attachments to the MT lattice, but the dynamic instability at the plus ends is markedly increased. This results in a high turnover of the MT lattice proximal to the plus ends (the ‘end zone’), as a result of the elevated frequency of catastrophe and rescue events. Presumably these changes in kMT dynamics prevent the establishment of regular kinetochore oscillations (see Discussion for detail). Moreover, the MT lattice distal to the end zone becomes hyperstable, causing an interruption of poleward MT flux, because MTs can no longer undergo treadmill.

Figure 8 Model for the function of the CENP-A NAC/CAD in controlling kMT dynamics. (a) Schematic view of the human kinetochore–MT interface showing the outer KMN and Ska complexes as well as the inner CENP-A NAC/CAD complex. Whereas the Ndc80/Nuf2 and KNL-1 subunits and Ska1/Ska2 subunits are essential for MT attachment, CENP-A NAC/CAD regulates the turnover of kMT plus ends, by controlling dynamic instability. There is a differential stoichiometry of subunits within the CENP-A NAC/CAD complex that is dependent on whether a sister kinetochore is bound to growing or shrinking MTs. The sister kinetochore bound to growing microtubules contains high levels of the CENP-H, CENP-I, CENP-K, CENP-M complex (red), whereas the sister kinetochore bound to shrinking MTs contains low levels of this complex (green). (b) Explanatory model for the lack of correlation between kMT stability and plus-end MT turnover. In control cells, kMT plus ends show low rates of dynamic instability, allowing the continual incorporation of new tubulin heterodimers. In contrast, in the absence of the CENP-A NAC/CAD complex, kinetochores still bind and stabilize kMTs through attachments to the MT lattice, but the dynamic instability at the plus ends is markedly increased. This results in a high turnover of the MT lattice proximal to the plus ends (the ‘end zone’), as a result of the elevated frequency of catastrophe and rescue events. Presumably these changes in kMT dynamics prevent the establishment of regular kinetochore oscillations (see Discussion for detail). Moreover, the MT lattice distal to the end zone becomes hyperstable, causing an interruption of poleward MT flux, because MTs can no longer undergo treadmill.
could be a part of a feedback mechanism that is predicted by modelling to be necessary for the emergence of chromosome oscillations\(^{(48)}\). It will therefore be crucial to determine whether these changes respond only to evolving MT dynamics or whether they have a more proactive function in the regulation of kinetochore oscillations.

**METHODS**

Methods and any associated references are available in the online version of the paper at [http://www.nature.com/naturecellbiology/](http://www.nature.com/naturecellbiology/)

**ACKNOWLEDGEMENTS**

We thank the ETH Light Microscopy Centre and Yves Barral for microscopy support; Anna Akhmanova, Helder Maiato, Michel Steinmetz, Daniel Gerlich, Erich Nigg, Stephan Diekmann and Jennifer Lippincott-Schwarz for reagents; Jason Swedlow, Gaudenz Danuser for the joint development of the kinetochore tracking assay; Satyarebala Borusu for the EGFP–CENP-O cell line; Jennifer Winter for initial photoactivation observations; Itsaso Olasagasti for helping with the CENP-I assay; Satyarebala Borusu for the EGFP–CENP-O cell line; Jennifer Winter for preparing the photoactivation experiments and Yves Barral, Monica Gotta, Helder Maiato, Jonathon Pines and members of the Barral, MacInnis and Meraldi laboratories for helpful discussions. Work in the MacInnis laboratory was supported by Marie Curie Cancer Care (A.D.M. and C.P.S.) and by a Fundação para a Ciência e Tecnologia fellowship (C.P.S.). A.C.A. is a member of the Life Science Zurich Graduate School in Molecular Life Sciences. Work in the Meraldi laboratory (A.C.A., R.H. and P.M.) was supported by a SNF-Förderungsfonds and a EYRIY1 award.

**AUTHOR CONTRIBUTIONS**

Project conception, planning and data analyses were performed by A.C.A., C.P.S., A.D.M. and P.M. A.C.A. performed all experiments except the following: C.P.S. performed the photoactivation experiments and the biochemical experiments. R.H. generated the photoactivatable GFP–α-tubulin/H2B–mRFP cell line. E.W. and M.L. measured Aurora A activity. I.K. and M.A. contributed to the biochemical experiments. The manuscript was prepared by A.D.M. and P.M. with contributions by A.C.A. and C.P.S.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

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1. Cheeseman, I. M., Chappie, J. S., Wilson-Kubalek, E. M. & Desai, A. The conserved reprintsandpermissions/evolving MT dynamics or whether they have a more pro-active function in the regulation of kinetochore oscillations\(^{(49)}\). It will could be part of a feedback mechanism that is predicted by modelling to be necessary for the emergence of chromosome oscillations\(^{(48)}\). It will therefore be crucial to determine whether these changes respond only to evolving MT dynamics or whether they have a more proactive function in the regulation of kinetochore oscillations.

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METHODS

Cell culture, short interfering RNA (siRNA) transfection, and drug treatments. HEK cells were grown at 37 °C in DMEM medium containing 10% FCS, 100 U ml−1 penicillin and 100 µg ml−1 streptomycin with 5% CO2 in a humidified incubator. The stable HEK EGFP–CENP-A and HEK EGFP–CLASP1 cell lines were grown as described previously29,30. To establish the stable HEK cell line expressing histone H2B–mRFP and photoactivatable GFP–α-tubulin, H2B–mRFP cells were transfected with a photoactivatable GFP–α-tubulin-IRE6-puro vector by using FuGENE6 (Roche), and positive cells were selected in the presence of 500 ng ml−1 puromycin. The cell line was maintained in DMEM containing 10% FCS, 100 U ml−1 penicillin, 100 µg ml−1 streptomycin, 250 ng ml−1 puromycin and 500 µg ml−1 G418. To obtain the stable cell line EGFP–CENP-I and EGFP–CENP-Q, HeLa cells were transfected with an EGFP–CENP–I-RES-puro or EGFP–CENP–O-RES-puro vector by using FuGENE6 (Roche). Both cell lines were selected and grown in DMEM containing 10% FCS, 100 U ml−1 penicillin, 100 µg ml−1 streptomycin and 500 ng ml−1 puromycin. Live-cell imaging experiments were performed at 37 °C in Lab-Tek II chambers (Thermo Fisher Scientific) with Leibovitz L-15 medium containing 10% FCS. Short RNA-mediated interference oligonucleotides (Qiagen or Invitrogen; control (LaminA)36, CENP-H (oligonucleotides 1 and 2)3), CENP-L, CLASP1 (ref. 29), MCAK, CENP-P (5′-GAACCTGGTAGGACTGCTTGGAAT-3′), EB1 (ref. 52) and Nuf2R (ref. 54) were transfected as described and analysed 48 or 72 h after transfection29. The most critical CENP-H experiments were repeated with a second CENP-H siRNA oligonucleotide, which gave qualitatively the same results (Supplementary Information, Fig. S4). All siRNA treatments were validated by immunofluorescence or immunoblotting (Supplementary Information, Fig. S5). For drug treatments, HEK cells were treated with 1 µM MG132 for 1 h, 1 µM Taxol for 20 min, or 1 µg ml−1 nocodazole for 1 h before fixation for immunofluorescence. Transient transfection of EGFP–CLASP2 (ref. 56) was performed with FuGENE 6 (Roche).

Antibody production. Rabbit antibodies were raised against Rubredoxin-MCAK expressed in E. coli and purified from inclusion bodies (NeoMPS). Peptide antibodies were raised in rabbits against CCRACEDPPAPWEEKSRLQVK (CENP-P), CSRSPEDFYFGATPLQKREL (CENP-L) and CKKNAQQLPKRRKQDDNNE (CENP-Q) peptides (Pepceuticals). Rabbit anti-CENP-P antibodies were affinity-purified against the immunizing peptide with the use of a Microlink Peptide Coupling Kit (Thermo Scientific).

Immunofluorescence microscopy. Cells were fixed at 20°C for 8 min in 20 mM PIPES pH 6.8 containing 10 mM EGTA, 1 mM MgCl2, 0.2% Triton X-100 and 4% formaldehyde. For cold-stable assays, cells were incubated on ice-cold medium 10–30 min before fixation. The following primary antibodies were used: rabbit anti-MCAK (1:2,000 dilution; this study), mouse anti-EB1 (1:500; BD Biosciences), rabbit anti-CENP-H (1:4,000)3, rabbit anti-CENP-I (1:1,000)3, rabbit anti-CENP-O (1:500)3, rabbit anti-CENP-P (1:500; this study), rabbit anti-Nuf2R (1:1,000)3, mouse anti-CENP-A (1:1,000; Abcam), mouse anti-a-tubulin (1:10,000; Sigma-Aldrich), CREST antisera (1:400; Antibodies Incorporated), rabbit anti-α-tubulin (1:1,000; Sigma-Aldrich), rabbit anti-CENP-B (1:1,000), rabbit anti-CENP-K (1:1,000), rabbit anti-CENP-I (1:1,000; this study), mouse anti-CENP-C (1:1,000; Abcam), rabbit anti-CENP-F (1:2,000; Novus Biologicals), mouse anti-p150Glued (1:500; BD Biosciences), rabbit anti-Kif18a (1:1,000; Bethyl Laboratories), rabbit anti-SkA1 (1:500)3, rabbit anti-Bub1 (1:8,000)3, mouse anti-BubR1 (1:1,500; Abcam) and rabbit anti-Sgo1 (1:500; Abcam). Cross-absorbed secondary antibodies (Invitrogen) were used. Three-dimensional image stacks of mitotic cells were acquired in 0.2-µm steps using a 60× or 100× oil-immersion 1.4 numerical aperture objective lens on an Olympus DeltaVision microscope (Applied Precision, LLC) equipped with a DAPI–fluorescein isothiocyanate (FITC)–rhodamine/TRC75 filter set (Chroma) and a CoolSNAP HQ2 camera (Roper Scientific). The three-dimensional image stacks were deconvolved with SoftWorx (Applied Precision, LLC) and mounted in figures with Photoshop and Illustrator (Adobe). For quantitative measurements, signals were determined and quantified with SoftWorx as described in Supplementary Information, Fig. S3.

Live still frames and live-cell imaging. Fluorescence time-lapse imaging of the EGFP–CENP-A and EGFP–CENP–O cell lines was recorded with a 100× 1.35 numerical aperture objective on an Olympus DeltaVision microscope (Applied Precision, LLC) equipped with a CoolSNAP HQ2 camera (Roper Scientific) and an EGFP filter set (Chroma) at a temporal resolution of 15 s for a total duration of 3 min. For each time point 20 Z-sections 0.5 µm apart were acquired. Live still frames were assembled in Adobe Illustrator by manually tracking sister-kinetochore pairs, cropping them out and adjusting the intensity levels (including gamma function) in Adobe Photoshop to compensate for photobleaching. These treatments were always applied to the whole image; that is, both sister kinetochores in a pair were subjected to the same adjustments.

Photoactivation experiments. Photoactivation experiments were performed on bipolar metaphase spindles (identified by the H2B–mRFP signal) as described previously with a 50-ms pulse from a 406-nm laser (20%) on a DeltaVision RT microscope equipped with a quantifiable laser module (Applied Precision, LLC). Fluorescence images were captured every 10 s for 530 s with a 100× oil-immersion 1.4 numerical aperture objective and a FITC filter set (Applied Precision, LLC). Fluorescence intensities were measured in a 20-pixel × 20-pixel area with SoftWorx (Applied Precision, LLC) over the first 200 s of each movie. Background spindle signal was subtracted for each time frame by measuring the same pixel area on the opposite side of the photoactivated spindle. Cells that underwent anaphase in this period were discarded, ensuring that all measurements were taken from cells in late prometaphase. The values were corrected for photobleaching by determining the percentage of fluorescence loss during the first 200 s in activated cells treated with 10 µM Taxol. The average data were fitted to a double-exponential curve I = P1 exp(−k1t) + P2 exp(−k2t), where I is the proportion of the initial fluorescence intensity, P is the proportion of fluorescence decay due to the fast (t) or slow (s) process, k is the rate constant for fluorescence decay of the fast (t) or slow (s) process, and t is time. Curve fitting was performed with KaleidaGraph (Synergy Software). The initial parameters P, k1, k2, P2 and k were set to 1, 0.1, 0.2 and 3.3 × 10−3, respectively, on the basis of previous results. The turnover half-time (t1/2) for each process was calculated as ln(2)/k for each fast and slow process.

Kinetochore tracking assay. For the kinetochore tracking experiments, fluorescence time-lapse imaging of an EGFP–CENP-A cell line was recorded with a 100× 1.35 numerical optical objective on an Olympus DeltaVision microscope (Applied Precision, LLC) equipped with a CoolSNAP HQ2 camera (Roper Scientific) and an EGFP filter set (Chroma) at a temporal resolution of 7.5 s and subjected to the kinetochore tracking analysis35. For each time-lapse movie, the position of the metaphase plate was estimated by fitting a plane to the calculated kinetochore positions. To characterize the dynamics of individual sister-kinetochore pairs located on the metaphase plate over time, we followed sister-kinetochore centre position along the normal to the metaphase plate (spindle axis). The autocorrelation function of sister-kinetochore movements along the spindle axis yielded the periodicity of sister-kinetochore oscillations. The displacement intervals correspond to the duration between consecutive directional switches of sister-kinetochore pairs. The average speed of sister kinetochores along the spindle axis was calculated as the standard deviation of the distribution of all sister-kinetochore frame-to-frame movements. The metaphase plate thickness was calculated as the standard deviation of the distribution of aligned sister centre positions along the normal to the metaphase plate.

FRET-based sensor for Aurora B activity. HEK cells were transfected first with 200 nM CENP-H siRNAs by using Oligofectamine (Qiagen), and then 24 h later with centromere-targeted Aurora B phosphorylation sensor36 by using FuGENE6 (Roche). Cells were imaged live 48 h after the sensor transfection, and mitotic cells were selected randomly. Imaging and analysis were performed as described previously. For treatment with the Aurora B kinase inhibitor ZM41, cells were treated with 2 µM for 1 h before imaging. For immunofluorescence staining, cells were fixed for 10 min at −20°C with precooled methanol. Images of fixed cells were acquired with the same spinning-disk confocal as for the live-cell imaging.

Immunoblotting. Whole-cell lysate preparation and immunoblotting were performed as described3, using rabbit anti-CLASP1 (1:500 dilution)3, mouse anti-a-tubulin (1:10,000; Sigma-Aldrich) and rabbit anti-CENP-Q (1:500; this study) and anti-mouse or anti-rabbit horseradish peroxidase-conjugated secondary antibodies (Amersham).
Protein purification, mass spectroscopy and MT-binding assays. Full-length CENP-A NAC/CAD genes were amplified from cDNAs (GENEART) and cloned into E. coli expression vectors (Novagen). The genes for CENP-Q and CENP-P were inserted into pRSFDuet-1 with an amino-terminal His-tag on CENP-Q. CENP-S and CENP-O genes were inserted into pCDFDuet-1, and CENP-H and CENP-U genes into pACYCDuet-1. The plasmids were co-transformed into BL21-CodonPlus-(DE3) E. coli and expression was induced with 0.5 mM IPTG, shaking overnight at 15 °C. Cell pellets were resuspended in 50 mM Tris-HCl pH 7.0, 100 mM NaCl, 30 mM imidazole and 5 mM 2-mercaptoethanol and lysed with 0.1 mg ml⁻¹ lysozyme for 1 h at 4 °C. Extracts were clarified at 16,000 r.p.m. for 40 min at 4 °C. Clarified extracts were purified with a Ni²⁺-NTA column (Qiagen) and the proteins were eluted with a 500 mM imidazole gradient. Proteins in each band were identified by trypsin digestion and microcapillary liquid chromatography–tandem mass spectrometry (MC–MS/MS) at the FingerPrints Proteomics Facility, University of Dundee. Hydrodynamic analysis of CENP-Q was performed as described⁵⁰, except that we used a Superose 12 10/300 GL column (GE Healthcare) for the size-exclusion chromatography, and all the assays were performed in 50 mM Tris-HCl pH 7.0, 100 mM NaCl, 5 mM 2-mercaptoethanol buffer. MT pelleting assays were performed as described previously⁵¹, except that we used BRB80 buffer (80 mM PIPES pH 6.8, 1 mM MgCl₂, 1 mM EGTA) containing 50 mM NaCl. The concentration of CENP-Q and tubulin in µM used in the MT pelleting assays was calculated as follows: 

\[
[A] = \frac{A_\text{abs}}{\varepsilon},
\]

where the absorption coefficient (ε) for CENP-Q = 8.25 × 10⁻³ and ε for tubulin = 0.10583. The concentrations were confirmed by Coomassie blue staining and comparison with a BSA standard curve. To obtain the CENP-Q–MT binding affinity parameters, the amounts of CENP-Q bound to MT (corrected for any insoluble material found in the fraction lacking MTs) and not bound to MT were obtained by quantification of the MT pelleting assays with Quantity One software (Bio-Rad Laboratories). The average data obtained were fitted to a hyperbolic function

\[
y = \frac{B_{\text{max}}x}{K_x + x},
\]

where y is the amount of CENP-Q bound to MT and x is the amount of CENP-Q not bound to MT; obtained previously. \( B_{\text{max}} \) is the maximal binding capacity. Curve fitting was performed with KaleidaGraph (Synergy Software).

52. Draviam, V. M., Shapiro, I., Aldridge, B. & Sorg, P. K. Misorientation and reduced stretching of aligned sister kinetochores promote chromosome missegregation in EB1- or APC-depleted cells. EMBO J. 25, 2814–2827 (2006).
Figure S1 CENP-H depletion impairs the localization of the whole CENP-A NAC/CAD complex. (a) Representative images of cells treated with siControl or siCENP-H RNAs stained with CENP-A (green) or CREST antisera (red) and CENP-H, CENP-I, CENP-P, CENP-O, CENP-N, CENP-K or CENP-L antisera (red) or CENP-C (green). Insets show higher magnification views of a single kinetochore. Scale bar = 10 µm.
Figure S2 CENP-H depletion does not have affect the binding of other key kinetochore proteins. (a-g) Representative images of siControl or siCENP-H RNA-treated cells stained with CREST (red) and p150Glued (a), CENP-F (b), Kif18a (c), Bub1 (d), BubR1 (e), Sgo1 (f) or Ska1 (g) antisera (green). Insets show higher magnification views of a single kinetochore. Scale bars = 10 µm.
**Figure S3** Immuno-fluorescence quantifications. (a) Scheme for the quantification of the intensity of CENP-H, CENP-I, CENP-O and CENP-P kinetochores. Intensities were normalized with the poles for each cell. (b) Scheme for the quantification of the intensity of EGFP-CLASP1 at the kinetochores. Intensities were normalized with the spindle poles. (c) Scheme for the quantification of the intensity of EB1 at kinetochores. Intensities were normalized with the spindle intensity. (d) Scheme for the quantification of the intensity of acetylated α-tubulin. The intensities were normalized with the total α-tubulin intensity. Scale bar = 10 µm.
Figure S4 Key experiments with an alternative CENP-H siRNA oligonucleotide (siCENP-H-2) confirm the results of this study (siCENP-H). (a) Autocorrelation function of translational sister-kinetochore movements along the spindle axis (kinetochore oscillation) of siControl (black line; same as in Figure 1b) and siCENP-H-2 (red line). The autocorrelation function was calculated by combining all aligned sister-kinetochore pairs for each condition (see Supplementary Table 2). (b-c) Histograms of the mean interval time between directional switches of the sister-kinetochore pairs along the spindle axis of siControl (black bars, same as in Figure 1b) and siCENP-H-2 (red bars). For each condition, the mean values and SDs are indicated. (d) Average sister-kinetochore pair speed along the spindle axis of siControl (black bar; same in Figure 1b) and siCENP-H-2 (red bar). Error bars represent SD based on n = independent experiments. (e) Width of the metaphase plate of siControl (black bar; same as in Figure 1b) and siCENP-H-2 (red bar). Error bars represent SD based on n = independent experiments. (f) Representative images of siControl and siCENP-H-2 cold-treated cells for 10 or 30 min stained with CREST antisera (kinetochores, red) and α-tubulin antibodies (MTs, green). (g) Fluorescence intensity decay of the photoactivated regions over time in siControl (black; same as in Figure 2) and siCENP-H-2 (red) treated PAGFP-α-tubulin/H2B-mRFP cells. Curve fitting indicated that the decay in CENP-H-2 depleted cells fitted to a curve with single exponential with the indicated half-life. Error bars represent SD. (h) Representative images of HeLa cells stably expressing EGFP-CLASP1 treated with siControl or siCENP-H-2 RNAs and stained with CREST antisera (kinetochores; red). Scale bars = 10 µm.
**Figure S5** siRNA depletions and antibody validations (a, b, d, e, g, h, j, k). Representative images of siControl and siCENP-H (a), siCENP-P (b), siCENP-O (d), siCENP-L (e), siNu2R (g), siMCAK (h), siNnf1R (j) or siEB1 (k) RNA-treated cells stained with CREST or CENP-A (red) and CENP-H (a), CENP-P (b), CENP-O (d), CENP-L (e), Nu2R (g), MCAK (h), Nnf1R (j) or EB1 (k) antisera (green). Insets show higher magnification views of a single kinetochore. (c, f, i) Immunofluorescence quantification of CENP-P (c), CENP-L (f) and MCAK (i) levels on aligned kinetochores after treatment with siControl and siCENP-P, siCENP-L or siMCAK RNAs from images such as shown in (b, e, h). Error bars indicate SEM (l) Validation of CLASP1 depletion. Immunoblots of whole cell lysates treated with siRNAs as indicated and probed with CLASP1 or α-tubulin antibodies are shown.
**Figure S6** Top-bottom gels of cropped immunoblots (a) Immunoblot of 5-40% glycerol gradient shown in Figure 7d probed with anti-CENP-Q antibodies. (b) Validation of CLASP1 depletion. Immunoblots of whole cell lysates treated with siRNAs as indicated and probed with CLASP1 or α-tubulin antibodies are shown. The cropped version is shown in Supplementary Figure S5l.
Supplementary movie legends

Supplementary Video S1: Poleward MT flux and tubulin turnover following photoactivation of PA-GFP-α-tubulin in a Control-depleted cell.
Supplementary Video S2: Poleward MT flux and tubulin turnover following photoactivation of PA-GFP-α-tubulin in a CENP-H-depleted cell.
Supplementary Video S3: Oscillating sister-kinetochore pair in an EGFP-CENP-I stable cell line.
**Supplementary Tables**

**Supplementary Table 1.** Qualitative levels of EB1 and CLASP1 at the kinetochores

<table>
<thead>
<tr>
<th>Protein/Condition</th>
<th>siControl</th>
<th>siNuf2R</th>
<th>siNuf1R</th>
<th>siCENP-H</th>
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<tr>
<td>EB1</td>
<td>+++&lt;sup&gt;a&lt;/sup&gt;</td>
<td>+++</td>
<td>++&lt;sup&gt;d&lt;/sup&gt;</td>
<td>++</td>
</tr>
<tr>
<td>CLASP1</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
<td>-&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>as determined by immunofluorescence  
<sup>b</sup>no signal  
<sup>c</sup>weak signal  
<sup>d</sup>reduced signal compared to control cells  
<sup>e</sup>normal signal

**Supplementary Table 2.** Number of imaged cells and sister kinetochore pairs used for the kinetochore tracking analysis

<table>
<thead>
<tr>
<th>Condition</th>
<th>Nº of cells</th>
<th>Nº sister KT pairs</th>
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<tr>
<td>siCENP-H</td>
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<tr>
<td>Fixed Cells</td>
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