## SUPPLEMENTARY EXPERIMENTAL PROCEDURES

### Primary antibody dilutions

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Source</th>
<th>Dilution</th>
<th>Localization</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human CREST anti-sera</td>
<td>Antibodies Incorporated</td>
<td>1:500</td>
<td>Kinetochores</td>
</tr>
<tr>
<td>Mouse anti-Ndc80</td>
<td>Abcam</td>
<td>1:1000</td>
<td>Kinetochores</td>
</tr>
<tr>
<td>Rabbit anti-Nuf2R</td>
<td>(Meraldi et al., 2004)</td>
<td>1:500</td>
<td>Kinetochores</td>
</tr>
<tr>
<td>Mouse anti-α-tubulin</td>
<td>B-5-1-1 (Sigma)</td>
<td>1:10,000</td>
<td>Spindle</td>
</tr>
<tr>
<td>Rabbit anti-γ-tubulin</td>
<td>T 3559 (Sigma)</td>
<td>1:1000</td>
<td>Centrosomes and spindle</td>
</tr>
<tr>
<td>Rabbit anti-CENP-E</td>
<td>(Meraldi et al., 2004)</td>
<td>1:1500</td>
<td>Chromosome passenger</td>
</tr>
<tr>
<td>Mouse anti-CENP-A</td>
<td>Abcam</td>
<td>1:4000</td>
<td>Kinetochores</td>
</tr>
<tr>
<td>Rabbit anti-Mcm21R</td>
<td>(McAinsh et al., 2006)</td>
<td>1:2000</td>
<td>Kinetochores</td>
</tr>
<tr>
<td>Rabbit anti-Nnf1R</td>
<td>(McAinsh et al., 2006)</td>
<td>1:4000</td>
<td>Kinetochores</td>
</tr>
<tr>
<td>Rabbit anti-Aurora B</td>
<td>This study</td>
<td>1:2000</td>
<td>Chromosome passenger</td>
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<tr>
<td>Preimmune</td>
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<td>None</td>
</tr>
<tr>
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<td>Kinetochores</td>
</tr>
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<tr>
<td>Preimmune</td>
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<tr>
<td>Rabbit anti-CENP-H.2</td>
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<td>1:2000</td>
<td>Kinetochores</td>
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### List of siRNA oligonucleotide sequences

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<tr>
<th>Protein</th>
<th>name</th>
<th>siRNA sequences</th>
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<tr>
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</tr>
<tr>
<td>Nnf1R (oligo 3)</td>
<td>sNnf1R-3</td>
<td>(McAinsh et al., 2006)</td>
</tr>
<tr>
<td>Nuf2R</td>
<td>sNuf2R</td>
<td>(Meraldi et al., 2004)</td>
</tr>
<tr>
<td>Mcm21R^{CENP-O} (oligo 1)</td>
<td>siMcm21R</td>
<td>(McAinsh et al., 2006)</td>
</tr>
<tr>
<td>Aurora B</td>
<td>siAurora B</td>
<td>(Meraldi et al., 2005)</td>
</tr>
<tr>
<td>Mcm21R^{CENP-D} (oligo 2)</td>
<td>siMcm21R-2</td>
<td>(McAinsh et al., 2006)</td>
</tr>
<tr>
<td>CENP-H (oligo 1)</td>
<td>siCENP-H</td>
<td>CAG AGA GGA UAA AGA UCA UAC GAC A</td>
</tr>
<tr>
<td>CENP-H (oligo 2)</td>
<td>siCENP-H-2</td>
<td>CAA CAU GUG UUC CAG AAC CUU AUU U</td>
</tr>
<tr>
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<td>siChl4R</td>
<td>AAC UAC CUA CGU GGU GUU CUA</td>
</tr>
<tr>
<td>Chl4R^{CENP-N} (oligo 2)</td>
<td>siChl4R-2</td>
<td>AAA UAU GCA AUU UCA UCA GCA</td>
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</table>
CENP-I (oligo 1)    | stCENP-I    | AAC AAA CCA UUU CGU GTG AGA  
CENP-I (oligo 2)    | stCENP-I-2  | AAG AAG GUG UGU GAC AUA UAU  
CENP-I (oligo 3)    | stCENP-I-3  | UAU UCC UGU GAA GCA UUC CUG UAU A  
CENP-I (oligo 4)    | stCENP-I-4  | GAC CAU CUA GCG CAG CUC UUC UUU A  
Sim4RCENP-K(oligo 1)| stSim4R     | AAG AUC UGG GUG CCA UCA UUU  
Fta1RCENP-L (oligo 1)| stFta1R | UUG AAA CAC GAA CUA AUC UUG UGG C  
Fta1RCENP-L (oligo 2)| stFta1R-2  | CCA UUA UGU GGC UAC UUG UGA AUU U  

**Hydrodynamic analysis**

Mitotic cell extracts were prepared from HEK293 cells treated with 100 ng/ml nocodazole for 16 h as previously described (Kline et al., 2006). For size exclusion chromatography (SEC) 5 mg mitotic cell extract was loaded on a HiPrep 16/60 Sephacryl S-500 HR column (Amersham) equilibrated in H150 buffer (50 mM HEPES, 150 mM KCl, 1 mM EDTA, 1 mM MgCl₂, pH 7.9) at 4°C. For sedimentation velocity ultracentrifugation 1 mg mitotic cell extract was loaded onto a 5%-40% (v/v) 5 ml glycerol density gradient layered in 13 x 51 mm polycarbonate centrifuge tubes (Beckman) using the Biocomp Gradient Master (Biocomp instruments). Gradients were centrifuged at 45,000 rpm for 14.5 h at 4°C in an AH-650 rotor (Sorvall). Fractions of 1 ml (SEC) or 200 μl (glycerol gradient) were collected, precipitated with 15% (v/v) trichloroacetic acid, washed with acetone and analyzed by immunoblotting with antibodies against proteins of interest. Calibration of gradients and SEC was done using the known sedimentation coefficients and Stokes radii of albumin, ovalbumin, chymotrypsin, catalase, aldolase and thyroglobulin (Amersham). The native molecular weight and shape (frictional coefficient) of protein complexes was calculated using established equations (Harding and Colfen, 1995; Schuyler and Pellman, 2002).

**Yeast 2-Hybrid Analysis**
Yeast 2-Hybrid Analysis was carried out using the Matchmaker™ Two-Hybrid System 3 (Clontech). Chl4R, Sim4R and Mcm21R were PCR amplified from pMC38 (EGFPC1-Chl4R) pMC41 (pDNR-Sim4R, Open Biosystems cDNA clone MGC12540), pMC56 (pDONOR207-Mcm21R), respectively, and cloned into pGBKKT7 and/or pGADT7. CENP-H plasmids were a gift from T. Fukagawa. pGBKKT7 plasmids were transformed into AH109 (Clontech) and pGADT7 plasmids were transformed into Wild Type W303 (ade2-101). Yeast strains were mated, in pair-wise combinations, on YPD plates at 30°C and diploids selected on SC–leu-trp plates. Three independent colonies were picked, grown to OD=1 and their growth tested by spotting 5-fold serial dilutions onto SC-leu-trp-his-ade and SC-leu-trp plates. Plates were then incubated for 2 days at 30°C. To check expression levels, whole cell extracts were prepared from haploid strains and analyzed by SDS-PAGE and immunoblotting. Primary antibodies used were monoclonal anti-c-myc at 1:1,000 and Monoclonal Anti-HA at 1:5,000 (both Sigma, UK). Detection kits used were Super Signal FEMTO kit (Perbio Science UK Ltd.).

SUPPLEMENTARY REFERENCES


Figure S1: Multiple sequence alignments of the CENP-M (A) CENP-P (B) and CENP-Q families (C). Schematic drawing above the alignment indicate the length of the *S. cerevisiae/S. pombe* and human proteins and the percentages denote the degree of similarity of successive sequence blocks (black boxes) within fungi and metazoa. White letters on black denote identical residues, white letters on green, identical residues in ≥80% of the organisms and black letters on green, similar residues in ≥80% of the organisms.
Figure S2
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**Figure S2**: Multiple sequence alignments of the CENP-S (A) CENP-T (B) and CENP-U families (C). Schematic drawing above the alignment indicate the length of the *S. cerevisiae/S. pombe* and human proteins and the percentages denote the degree of similarity of successive sequence blocks (black boxes) within fungi and metazoa. White letters on black denote identical residues, white letters on green, identical residues in ≥80% of the organisms and black letters on green, similar residues in ≥80% of the organisms.
Figure S3: Characterization of anti-CENP-H.2 and anti-Chl4R.2 antisera. (A) Immunofluorescence images of interphase HeLa cells transfected with siLaminA or siChl4R and stained with DAPI (DNA; not shown), CREST antisera (kinetochores; red) and anti-Chl4R.2 antibodies (green). (B) Levels of Chl4R on kinetochores were determined from deconvolved 3D reconstructions of cells stained with DAPI (DNA), CREST antisera (kinetochores) and Chl4R.2 antisera. The intensity of the kinetochore signal was determined in interphase (I), prophase (P), prometaphase (PM), metaphase (M), anaphase (A) or telophase (T) relative to CREST after background correction (see experimental procedures for details). Profile for results with anti-Chl4R antibodies are shown for comparison. (C) Immunofluorescence images of interphase HeLa cells transfected with siLaminA or siCENP-H and stained with DAPI (DNA; not shown), CREST antisera (kinetochores; red) and anti-CENP-H.2 antibodies (green). (D) Levels of CENP-H and Chl4R on kinetochores were determined in control (siLaminA) and siMcm21R transfected cells from deconvolved 3D reconstructions of cells stained with DAPI (DNA), CREST antisera (kinetochores) and the corresponding antisera. The intensity of kinetochore signal was determined relative to CREST after background correction. Scale bar = 10 μm.
Figure S4: Representative images of interphase cells treated with: (A) control, siCENP-H, siCENP-H + siChl4R or siCENP-H + siNnf1R-3 and stained with anti-CENP-H antibodies (red). (B) control, siChl4R or siCENP-H + siChl4R and stained with anti-Chl4R (red) antibodies. (C) control, siNnf1R-3 or siCENP-H + siNnf1R-3 and stained with anti-Nnf1R (red) antibodies. All cells were also stained with CREST (green; to mark kinetochores) and DAPI (to mark DNA; not shown). Quantification of experiments in (A-C) is shown in Figure 3C and E. Scale bar = 10 μm.
Figure S5: (A) Representative images of interphase cells treated with control, siMcm21R, siCENP-H, siChl4R, siChl4R + siMcm21R, siCENP-H + siMcm21R or partial siCENP-H + siMcm21R RNAs and stained with CREST antisera (red; to mark position of kinetochores) and anti-Mcm21R (left row; green), anti-CENP-H (middle row; green) or anti-Chl4R (right row; green) antibodies. Scale bar = 10 μm (B) Immunoblots of lysates from cells transfected with siRNAs as indicated and probed with anti-Mcm21R, anti-CENP-H, anti-Chl4R and anti-tubulin antibodies.
Figure S6: Characterization of Aurora B, CENP-I, Sim4R and Fta1R antibodies and siRNAs. (A) Characterization of anti-Aurora-B antibodies: anti-peptide antibodies against human Aurora B (but not the corresponding pre-immune sera) recognized the inner centromere region (upper panel). This staining was specific, as treatment with an siRNA towards Aurora B eliminated the kinetochore signal, whereas a control siRNA had no effect (lower panel). Cells were stained with CREST (red), to mark position of kinetochores, and anti-AuroraB antibodies (green). Scale bar = 10 μm. Increased number of kinetochores in *siAuroraB* treated cells reflects the failure in cytokinesis following Aurora B inactivation. (B) Levels of CENP-I on kinetochores following treatment with 1 of 4 different siRNAs targeted towards CENP-I by quantitative immunofluorescence. (C) Representative images of cells treated with control (*siLaminA*) or *siCENP-I* RNAs and stained with CREST, to mark position of kinetochores, and anti-CENP-I antibodies. Scale bar = 10 μm. (D) Immunoblots of lysates from cells transfected with control siRNA or an siRNA targeted towards Sim4R and probed with antibodies as indicated. Arrow indicates where the Sim4R protein migrates. (E) Representative images of U2OS cells following transfection with control or *siSim4R* RNA and stained with CREST antisera and anti-Sim4R antibodies. (F) Immunoblots of lysates from a HEK293 cell line containing a stably integrated ProteinA-Flag-tagged Fta1R construct (called PAF:Fta1R) transfected with control, *siFta1R* or *siFta1R-2* RNAs and probed with anti-proteinA (anti-PAP) or anti-tubulin antibodies. (G) Immunoblots of PAF:Fta1R transfected with siRNAs as indicated and probed with anti-proteinA (anti-PAP) or anti-tubulin antibodies. (H) Percentage monopolar spindles in fixed cells treated with control, *siMcm21R, siMcm21R-2, siFta1R, siFta1R-2, siMcm21R + siFta1R and siMcm21R-2 + siFta1R* RNAs. Note that both Mcm21R and Fta1R siRNAs cause a monopolar spindle phenotype and that combination of Fta1R depletion with one of two independent siRNAs targeted towards Mcm21R does not rescue the monopolar phenotype.
Figure S7: Biochemical analysis of NDC80, MIND/Mis12, Mcm21R, Chl4R and CENP-H subcomplexes. (A-F) Hydrodynamic analysis of Ndc80, Nnf1R, CENP-H and Mcm21R kinetochore proteins. Whole cell extracts were prepared from nocodazole-arrested HEK293 cells and analyzed on a 5%-40% glycerol-density gradient (A, C) and a HiPrep 16/60 Sephacryl S-500 HR size exclusion chromatography column (B, D). Fractions were immunoblotted with anti-Ndc80, anti-Nnf1R, anti-CENP-H or anti-Mcm21R antibodies (E, F) and protein levels of Ndc80 (black line), Nnf1R (purple line), CENP-H (blue line) and Mcm21R (green line) quantified (A-D). (G) Immunoblots using whole cell extracts prepared by boiling cells directly in SDS buffer (lane 1) and soluble (lane 2) or insoluble fractions (lane 3) from clarified whole cell extracts prepared by breaking cells in liquid nitrogen (see materials and methods for details). Our hydrodynamic analysis (see Table S1) showed that, as previously reported, human Ndc80 ran as part of a 172 kDa complex (Ciferri et al., 2005), while Nnf1R was part of at least three distinct subcomplexes with predicted molecular weights ranging from 25 to 347 kDa (Kline et al., 2006). Our analysis also showed that Mcm21R was part of a 252 kDa subcomplex and CENP-H was found in a 43 kDa subcomplex which is potentially a dimer of CENP-H (Sugata et al, 2000). These subcomplexes were both distinct from each other and from NDC80 or MIND. We also note that Chl4R was found exclusively in the insoluble pellet fraction and was, unlike all other tested kinetochore proteins, undetectable in soluble extracts. These findings confirm that the CENP-A NAC/CAD is assembled from a set of soluble subcomplexes that are distinct from NDC80 and MIND.
**Figure S8: Mcm21R does not interact with CENP-H.** Yeast two-hybrid interactions between Mcm21R expressed as a binding domain (pGBK) fusion and CENP-H expressed as an activation domain (pGAD) fusion. As positive controls, Mcm21R is shown to interact with Sim4R (middle panel, third row) and CENP-H is shown to interact with CENP-H and Sim4R (bottom panel, third and fourth rows). A weak interaction was detected between Chl4R and CENP-H (bottom panel, second row). As negative controls, the empty pGAD and pGBK vectors were used. Interactions were assessed by growth on selective media (SC-his-leu-his-ade, right column). For control, growth on nonselective plates was used (sc-trp-leu, left column). Western analysis demonstrated that all constructs were expressed at similar levels in haploid yeast strains (data not shown).
**Supplementary table 1**  
McClelland, Borusu *et al.*

<table>
<thead>
<tr>
<th>Analyzed Protein</th>
<th>Predicted molecular mass (kDa)</th>
<th>Subcomplex</th>
<th>Svedberg coefficient ((10^{-13}s))</th>
<th>Stokes radius (Å)</th>
<th>Native molecular mass (kDa)</th>
<th>Presumed composition and mass (kDa)(^1)</th>
<th>Frictional coefficient f/fo</th>
<th>Axial ratio (a:b)</th>
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</thead>
<tbody>
<tr>
<td>Ndc80</td>
<td>74</td>
<td>NDC80 complex</td>
<td>5.0 ± 0.7</td>
<td>83.6 ± 6</td>
<td>172.0 ± 28</td>
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<tr>
<td>Nnf1R</td>
<td>25</td>
<td>MIND complex</td>
<td>6.4</td>
<td>131.25</td>
<td>at least three complexes ranging from 25 to 347 kDa</td>
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<td>N.D.</td>
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<td>51.1 ± 4</td>
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<td>20 ± 0</td>
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<td>Mcm21R/CENP-O</td>
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<td>Catalase(^3)</td>
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<td>Catalase</td>
<td>11.8 ± 0.3</td>
<td>46.5 ± 6</td>
<td>225.4 ± 26</td>
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<td>1.2 ± 0.1</td>
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\(^1\) Sum of the masses of the known subcomplex components  
\(^2\) N.D.: Not determined  
\(^3\) Included as a control (Horiike et al., 1983)