Figures and figure supplements

The pseudo GTPase CENP-M drives human kinetochore assembly

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**Figure 1.** Ablation of CENP-M perturbs kinetochore function. (A) Core kinetochore components. CENP-C and CENP-T/W may create independent connections between centromeres and outer kinetochores. Green lines indicate direct connections with centromeric DNA or chromatin. Black lines indicate recruitment dependencies. CENP-C binds directly to CENP-A and Mis12 complex (Przewloka et al., 2011; Screpanti et al., 2011; Kato et al., 2013). Figure 1. Continued on next page
CENP-T, together with CENP-W, S and X, may form a nucleosome-like structure interacting directly with DNA and the Ndc80 complex (Hori et al., 2008a; Gascoigne et al., 2011; Schleiffer et al., 2012; Nishino et al., 2013, 2012). Sub-complexes of CCAN subunits were inferred from reconstitution or from similarity of depletion phenotypes (see main text). (B) Representative immunofluorescence (IF) images showing endogenous CENP-M localization to kinetochores of HeLa cells in both interphase and mitosis. Kinetochores were visualized with CREST sera and DNA stained with DAPI. Insets show a higher magnification of regions outlined by the white boxes. Scale bar = 2 µm. (C) Whole cell protein extracts from HeLa cells treated with specific siRNAs (showed in D) were run on SDS-PAGE and immunoblotted for the indicated kinetochore proteins. Vinculin was the loading control. MWM, molecular weight marker. (D) HeLa cells depleted for CENP-M display significant chromosome congression defects. Following fixation, cells treated with CENP-M siRNA were imaged for endogenous CENP-M, CREST and DNA (DAPI). Scale bars = 2 µm. (E) CENP-M kinetochore levels from the experiment in D. Quantifications are expressed as normalized CENP-M/CREST fluorescence intensity ratios. Graphs and bars indicate mean ± SEM. (F) Quantification of chromosome congression defects in D. As a positive control, cells treated with 500 nM Hesperadin were scored for alignment defects. (G) Quantification of the percentage of mitotic cells in the experiment in D.

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Figure 1—figure supplement 1. Additional localization data.

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Figure 2. Characterization of the HIKM complex. (A) SEC elution profile of CENP-M with associated SDS-PAGE separations of peak fractions indicated by the horizontal bar under the profile. CENP-M (∼20 kDa) elutes as expected for a monomeric species. (B) Schematic representation of the primary sequence of CENP-H, CENP-I, CENP-K, and CENP-M. (C) SEC elution profile and SDS-PAGE separation of the CENP-H/K complex. CENP-H/K forms a 1:1 dimer (∼61 kDa) (Figure 2—figure supplement 1, panel B) but elutes near the 158 kDa marker, indicative of an elongated complex. (D) SEC elution Figure 2. Continued on next page
Figure 2. Continued

profile and SDS-PAGE separation of the CENP-H<sup>57<–281</sup>–KM complex. CENP-H<sup>57<–281</sup>–KM (~159 kDa) elutes near the 158 kDa marker, suggesting that the complex contains a single copy of each subunit. (E) Summary of cross-links. Intra-molecular cross-links are shown in blue and outside the ideal perimeter designed by the four subunits of the complex. Inter-molecular cross-links are shown as black lines. (F) CENP-H/His-CENP-K complex and His-CENP-H<sup>57<–281</sup>, both at 10 µM, form a stoichiometric complex as shown by co-elution from SEC runs and corresponding SDS-PAGE separations. (G) Lack of co-elution from SEC runs and SDS-PAGE analysis indicate that CENP-H/K<sup>57<–281</sup> complex and CENP-M do not bind.
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Figure 2—figure supplement 1  Additional biochemical characterization.
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Figure 3. A small G-protein fold in CENP-M. (A) Cartoon model of CENP-M\(^{1-171}\) in two orientations. (B) Cartoon model of Rab1A/GDP (PDB ID 4FMC). (C) Sequence alignment based on the structural superposition of CENP-M\(^{1-171}\) with Rab1A. Conserved elements of small G proteins are in yellow. A conserved residue involved in catalysis and targeted by activating mutations in Ras is in light blue. Secondary structure elements of CENP-M\(^{1-171}\) not Figure 3. Continued on next page.
present in Rab1A/GDP are in red, while those present in Rab1A/GDP but not in CENP-M1–171 are in green. (D) Experiments with N-methylantraniloyl (MANT) derivatives of GTP and ATP. Binding of MANT-GTP or MANT-ATP to the small GTPase Arl2 (‘Arl2’) or CENP-M (‘M’) was monitored at an emission wavelength of 440 nm (See Figure 3—figure supplement 1, panel D). The histogram shows the time-averaged fluorescence value after addition of the indicated proteins to a solution of the indicated MANT nucleotides, normalized against the time-averaged value prior to protein addition. Only the addition of Arl2 to MANT-GTP (or MANT-GDP, see Figure 3—figure supplement 1, panel D) gave a clear increase in signal indicative of a physical interaction of the MANT nucleotide with Arl2. (E) Unrooted maximum likelihood tree of 157 sequences. Shown are members of classical small GTPase families in many species, covering a wide range of evolutionary points. Gray names are reclassified families (Rojas et al., 2012). Bold names are human sequences. CENP-M sequences are pink. Uppercase indicates Uniprot code for proteins. 3-code labels are: Nve (Nematostella vectensis), Bfl (Branchiostoma floridae), Xtr (Xenopus tropicalis), and Cin (Ciona intestinalis). Numbers on the left of 3-code labels are accession numbers corresponding to the DOE Joint Genome Institute (JGI) database. Red and underlined names are Genbank gi identifiers found in iterative hmmer searches against non-redundant database. Acanth. castellanii indicates Acantarthamoeba castellanii, where * indicates that this entry is annotated as a RAS protein. Dicty. purpureum is Dictyostelium purpureum, Aae is Aedes aegypti, Aqu is Amphimedon queenslandica (sponge), and Clu is Clavispora lusitaniae (fungi). Red numbers within the tree indicate number of trees corresponding to more than 80% of statistical support for a given group, whereas black indicates values below 80%. Only representative numbers have been shown for clarity.

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Figure 3—figure supplement 1  Additional analyses of CENP-M (related to Figure 3).

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Figure 3—figure supplement 2. Comparison of CENP-M with Rab-like GTPases.
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Figure 4. Structural organization of the HIKM complex. (A) Cartoon representation of the CENP-I model generated by program I-TASSER (left), of the Importin-β/Ran complex (middle), and of a hypothetical structure between CENP-I and CENP-M modeled on the Importin-β/Ran complex (right). A scoring function (C-score) associated with I-TASSER models estimates accuracy of structure predictions. C-score is typically in a range from −5 to 2, where a higher score reflects a model of better quality. Both false positive and false negative rates are estimated to be below 0.1 when a C-score >−1.5 is displayed (Zhang, 2008). The CENP-I model is associated with a C-score of −1. (B) Representative class averages of the negatively stained HIKM complex. Figure 4—figure supplement 3 shows the complete set of class averages. Scale bar = 10 nm. (C) A 3D reconstruction of HIKM complex from negatively stained particles at ~22 Å resolution. Scale bar = 10 nm. (D) Summary of interactions in the CENP-HIKM complex. The central regions of CENP-H and CENP-K may form an extended parallel interaction, possibly through an α-helical arrangement, which interacts more or less co-linearly with the N-terminal region of CENP-I (I<sup>N</sup>). Additional globular domains may be present at the N- and C-termini of CENP-H and CENP-K. The entire sequence of CENP-I may fold as a helical solenoid. CENP-M does not interact with CENP-H/K and may bind near the concave surface of the predicted CENP-I solenoid, becoming largely buried. (E) siRNA depletion of endogenous CENP-M abrogates CENP-I kinetochore localization in HeLa cells. Representative cells displayed here are the same shown in Figure 1D, but with addition of CENP-I staining (left panels). Insets display a higher magnification of regions outlined by white boxes. Scale bars = 2 µm. (F) CENP-M and CENP-I kinetochore levels from the experiment illustrated in E. Quantification for CENP-M kinetochore levels are the same shown in Figure 1D and were performed as previously described. Graphs and bars indicate mean ± SEM. DOI: 10.7554/eLife.02978.013
Figure 4—figure supplement 1. Structural predictions on CENP-I orthologs.
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Figure 4—figure supplement 2. Conservation mapped on the CENP-I model.
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Figure 4—figure supplement 3. EM analysis.
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Figure 4—figure supplement 4. Fitting the CENP-I/M model in the EM density.
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Figure 5. CENP-M residues required for kinetochore targeting of CENP-I. (A) The CENP-M alignment identifies highly conserved residues (based on alignment in Figure 3-figure supplement 1, panel D), a subset of which (asterisks) is exposed at the surface of CENP-M. (B) Position of conserved residues shown in A on two opposite faces of the CENP-M surface. (C) After insect cell co-expression of indicated proteins, affinity purification with GST-CENP-M (if present) led to isolation of associated proteins shown, after SDS-PAGE separation, in the ‘beads’ fraction. Material eluted from beads was collected and shown in lanes labeled ‘elution’. Co-expression of all four CENP-HI57–CMA subunits (positive control) is necessary for identification of the CENP-HI57–CM complex on beads and in elution fraction. CENP-ML94A–L163E fails to assemble CENP-HI57–CM despite the expression of CENP-H, CENP-I57–CM, and CENP-K. (D) GFP-CENP-M+, but not GFP-CENP-MAZ, co-immunoprecipitates CENP-I, CENP-T and Mis12 from mitotic cells. Panels Figure 5. Continued on next page
represent the α-GFP co-immunoprecipitation analysis of protein extracts obtained from mitotic HeLa Flp-In T-REx cells stably expressing GFP, GFP-CENP-M\textsuperscript{wt} or GFP-CENP-M\textsuperscript{L94A+L163E} from an inducible promoter. Total protein extracts (Input) and immunoprecipitates (α-GFP IP) were run on SDS-PAGE and subjected to WB with indicated antibodies. Vinculin was used as a loading control. (E) Representative images of HeLa Flp-In T-REx cells treated with siRNA for endogenous CENP-M and expressing the indicated siRNA-resistant GFP-CENP-M fusions. Expression of GFP-CENP-M\textsuperscript{wt}, but not of GFP-CENP-M\textsuperscript{L94A+L163E}, rescues chromosome alignment defects and loss of CENP-I kinetochore localization observed upon depletion of endogenous CENP-M. Following fixation, cells were immunostained and imaged for GFP, CENP-I, CREST and DNA (DAPI). Insets show a higher magnification of regions outlined by white boxes. Scale bars = 2 µm. (F) Quantification, for experiment in E, of the CENP-I kinetochore levels normalized to CREST kinetochore signal. Graphs and bars indicate mean ± SEM. See ‘Materials and methods’ section for details on quantification.

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Figure 5—figure supplement 1. Stability of CENP-M mutant.

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Figure 5—figure supplement 2  Inducible expression and localization of CENP-M. 
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Figure 6. Significance of the CENP-M/CENP-I interaction for kinetochore assembly. (A–D) Representative images of the localization of kinetochore proteins in HeLa Flp-In T-REx cells treated with siRNA for endogenous CENP-M and expressing the indicated siRNA-resistant GFP-CENP-M fusions. Scale bars = 2 µm. (E) Quantification, for experiments A–D, of the kinetochore levels of the indicated proteins normalized to CREST. Graphs and bars indicate mean ± SEM. (F) Depletion of CENP-C abrogates kinetochore accumulation of CENP-T/W. Representative images of HeLa cells treated with Figure 6. Continued on next page
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Figure 6. Continued

siRNA for CENP-C or CENP-T and arrested in G2 with the Cdk1 inhibitor RO-3306 (‘Materials and methods’). Following fixation, cells were immunostained for CENP-C, CENP-T/W and CREST. DNA was stained with DAPI. Scale bars = 10 µm. (G) Quantification, for experiment F, of the kinetochore levels of the indicated proteins normalized to CREST kinetochore signal. Graphs and bars indicate mean ± SEM.
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Figure 7. Direct interaction of HIKM complex with the CENP-T/W complex. (A–D) GST or GST-HIKM baits were immobilized on beads and incubated with (A) CENP-T/W/S/X complex, (B) CENP-T<sup>452–C</sup>/W/S/X complex, (C) CENP-T<sup>452–C</sup>/W, or CENP-S/X. For each sample, both the input and the solid phase bound material (indicated as 'Pull-down') were analyzed by gel electrophoresis.

Figure 7. Continued on next page
Figure 7. Continued

('pull-down') are shown after separation by SDS-PAGE and staining with Coomassie brilliant blue. Note that full-length CENP-T and CENP-I<sup>57–C</sup> migrated indistinguishably. (E) Model of kinetochore assembly supported by our analysis. CENP-C and possibly CENP-N/L interact directly with the CENP-A nucleosome. The presence of CENP-C at the centromere is essential for the recruitment of CENP-T/W and CENP-HIKM complex. CENP-T/W and CENP-HIKM complex are co-dependent and interact physically with each other.

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