Figures and figure supplements

The quantitative architecture of centromeric chromatin

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Figure 1. CENP-A levels are regulated by mass-action. (A) Schematic of gene-targeting strategy that allowed for the creation of CENP-A knockout and fluorescent knock-in alleles. The region encoding the essential CENP-A targeting domain (CATD, Black et al., 2007) is indicated. (B) Quantitative immunoblots of CENP-A, HJURP, and Mis18BP1 in differentially targeted RPE cell lines. α-tubulin is used as a loading control. (C) Immunofluorescence images of same cell lines as in B. CENP-A intensity is represented in a heat map as indicated on the right. The fold difference ± SEM (n is biological
Figure 1. Continued

replicates) compared to wild-type RPE cells is indicated below. Scale bar: 10 μm. Note that in contrast to quantification of immunoblots, immunofluorescence detection of untagged and tagged CENP-A is directly comparable. (D) Quantification of centromeric CENP-A levels (from C) by immunofluorescence (IF) and total CENP-A levels (n = 4–9 independent experiments as in B) by western blot (WB). All cell lines expressing untagged CENP-A are normalized to CA−/− while those expressing tagged CENP-A are normalized to the centromeric CA−/− levels measured in C, as indicated by dashed lines. (E) Correlation of centromeric and total cellular CENP-A levels as measured in D. Dashed line represents a predicted directly proportional relationship with indicated correlation coefficients. Throughout, the average ± SEM is indicated. (F) Quantification of centromeric CENP-A levels in synchronized HeLa cells (based on anti-CENP-A staining) within a single cell cycle after transient transfection of indicated proteins. Asterisk indicates statistically significant increase compared to control or indicated transfections (one-tailed t test; p<0.05; n = 3); NS indicates no significant increase. Average ± SEM of three independent experiments is shown.

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Figure 1—figure supplement 1. CENP-A expression is the rate limiting factor for centromeric CENP-A levels.

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Figure 2. Human centromeres contain 400 molecules of CENP-A. (A) Schematic outline of strategy allowing for the quantification of the centromeric fraction of CENP-A compared to the total cellular pool. Scale bars: 5 μm. (B) Quantification of the centromeric fraction of CENP-A in CA<sup>−/−</sup> cells. Each circle represents one centromere; Figure 2. Continued on next page
Figure 2. Continued

circles on the same column are individual centromeres from the same cell. Dashed line indicates average of all centromeres. (C) Quantification of the centromeric fraction of CENP-A in indicated cell lines. Each square represents the average centromeric signal from one cell; squares on the same column are individual cells from the same experiment (Exp). **Figure 2—figure supplement 2** shows quantification of individual centromeres in CA<sup>−/−</sup> and CA<sup>−/−</sup>+OE cells. (D) Representative quantitative immunoblot of purified recombinant CENP-A and endogenous CENP-A from whole cell extracts (WCE). (E) Quantification of D. Solid line represents the best fit linear regression. Dashed line represents the amount of CENP-A from 150,000 cells. (F) Quantification of the total cellular CENP-A copy number. Each diamond represents one replicate experiment; measurement from E is indicated as a gray diamond. (G) Calculation of average CENP-A copy number per centromere (CEN) in wild-type RPE cells.

Throughout, the average ± SEM is indicated.

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**Figure 2—figure supplement 1**. Representative fluorescence lifetime imaging (FLIM) micrograph of a CENP-A-YFP expressing cell (left) and quantification of indicated cellular regions (right).

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Figure 2—figure supplement 2. Measurements of individual centromeres and CENP-A levels for different cell lines.
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Figure 2—figure supplement 3. Transfer efficiency of recombinant and cellular CENP-A.
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Figure 3. Centromeric CENP-A levels are equivalent between S phase and randomly cycling cells. (A) Cartoon depicting changes in cell morphology and nuclear levels of hCdt1(30/120)-RFP (in red) throughout the cell cycle (Sakaue-Sawano et al., 2008). Approximate timing of CENP-A expression (Shelby et al., 2000) and centromeric loading (Jansen et al., 2007) are indicated in orange and blue, respectively. The stage at which cells were analyzed to measure the centromeric fraction of CENP-A is indicated in green. (B) An example trace of a cell entering S phase (indicated by a sudden decrease in RFP levels) is shown. The centromeric fraction of CENP-A was measured at this point as outlined in Figure 2A. Peak expression is normalized to 100 and background fluorescence to 0. Micrographs of hCdt-1(30/120)-RFP at indicated timepoints are shown below. (C) As in Figure 2C. Orange squares represent cells that have passed the G1-S transition point, as indicated by decreasing levels of hCdt1(30/120)-RFP. Gray squares represent randomly cycling cells. No statistically significant differences (NS) were observed between randomly cycling cells and S phase cells.

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Figure 3—figure supplement 1  hCdt-1(30/120)-RFP expression allows for accurate determination of cell cycle stages and measurements of centromeric CENP-A ratios.
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Figure 4. Measurement of nuclear CENP-A confirms centromeric copy number. (A) As in Figure 2B, except that the centromeric fraction compared to total nuclear pool is indicated. Inset shows a representative image of a CA<sup>−/−</sup>+H2B-RFP cell (scale bar: 2.5 μm). (B) Quantitative immunoblot showing the soluble fraction and a dilution series from the insoluble fraction of CENP-A-YFP in CA<sup>−/−</sup>+H2B-RFP cells (left). Tubulin is used as a marker for the soluble fraction and H4K20me2 (exclusively found in chromatin, Karachentsev et al., 2007) for the insoluble fraction. Quantification of insoluble fraction of CENP-A is shown to the right. (C) Calculation of the average CENP-A copy number per centromere (CEN) in wild-type RPE cells, based on results from CA<sup>−/−</sup>+H2B-RFP cells. DOI: 10.7554/eLife.02137.011
**Figure 5.** Independent quantification methods confirm centromeric CENP-A copy number. (A) Stochastic fluctuation method: cartoon depicting inheritance and random redistribution of parental CENP-A nucleosomes onto sister chromatids during DNA replication. A hypothetical distribution of the absolute difference between the two sister centromeres, as well as the formula for calculating the fluorescence intensity per segregating unit ($\alpha$) are indicated on the right. (B) Representative image of mitotic CENP-A-YFP expressing cell. CENP-B staining allows for identification of sister centromeres. Blowup to the right represents a single slice of the boxed region showing that CENP-B is located in between the CENP-A spots of sister centromeres. (C) Frequency distribution of the difference between centromeric CENP-A copy number.

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Figure 5. Continued
CENP-A-GFP intensity of sister centromeres in CA<sup>δ</sup>-<sup>−</sup> cells. (D) Quantification of centromeric CENP-A-GFP based on the stochastic fluctuation method. Each circle represents one centromere; circles on the same column are individual centromeres from the same cell. Left y-axis indicates segregating CENP-A-GFP units in CA<sup>δ</sup>-<sup>−</sup> cells; right y-axis shows the conversion to minimum number of centromeric CENP-A molecules in CA<sup>δ</sup> (WT) cells. (E) Fluorescent standard method: representative fluorescence images of 4kb-LacO, LacI-GFP <i>S. cerevisiae</i> and human CA<sup>δ</sup>-<sup>−</sup> cells. (F) Quantification of fluorescence signals of LacI-GFP and CENP-A-GFP spots (n = 2 biological replicates). The left y-axis indicates the fluorescence intensity normalized to LacI-GFP; the right y-axis shows the conversion to maximum number of centromeric CENP-A molecules in wild-type cells. (G) Comparison of independent measurements for the centromeric CENP-A copy number (corrected for CA<sup>δ</sup> levels; Stoch. fluctuations = stochastic fluctuation method [Figure 5A]; Integr. fluorescence = integrated fluorescence method [Figure 2A]). Levels from all strategies are corrected for CA<sup>δ</sup> (WT) levels. Throughout, the average ± SEM and scale bars of 2.5 μm are indicated.

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Figure 5—figure supplement 1. Stochastic fluctuations of CENP-A segregation allows for copy number measurements.
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Figure 6. Reduction of CENP-A leads to a CENP-C, CENP-T, and Hec1 independent increase in micronuclei. (A) Quantification of centromeric CENP-A (from Figure 1), CENP-C, CENP-T, and Hec1 levels for indicated cell lines; n = 4 independent experiments in each case. Note that cell lines carrying tagged CENP-A have a slight, yet non-significant impairment in recruiting CENP-C, CENP-T, and Hec1. However, this does not correlate with the CENP-A levels themselves. Below, representative images of indicated antibody staining from CA<sup>+/+</sup> cells are shown. Representative images from all cell lines can be found in Figure 6—figure supplement 1. (B) Quantification of the fraction of cells containing micronuclei (MN) for indicated cell lines. Asterisk indicates statistically significant increase compared to wild-type (paired t test; p<0.05; n = 3–4 independent experiments [500–3000 cells per experiment per cell line]); NS indicates no significant difference. Throughout, the average ± SEM is indicated and dashed lines represent wild-type levels. Scale bars: 5 μm.

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Figure 6—figure supplement 1. Representative images for quantifications in Figure 6B.
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Figure 7. Centromere and cell specific distribution of CENP-A. (A, C, E) Representative micrograph of mitotic spreads for LacI-GFP::LacO expressing HCT-116 cells (A); wild-type HCT-116 cells (C); and PDNC-4 cells (E). Blowups show the chromosome containing the integrated Lac-array (A); the Y-chromosome (outline indicated; CENP-B negative) as well as an autosome (CENP-B positive) (C); and the neocentric chromosome 4, containing CENP-A (E).
Figure 7. Continued

2 pairs of ACA spots (staining both CENP-A and CENP-B), but only 1 pair of CENP-A spots (E, B, D, F). Quantification of CENP-A levels on the centromere of the chromosome containing the Lac-array (CEN-Lac; n = 29, B); the Y-chromosome (CEN-Y; n = 18, D); and neocentric chromosome 4 (NeoCEN-4; n = 39, F) of indicated cell lines compared to all other centromeres within the same cell (Other CENs; n = 1008, 620, and 1592, respectively). Median (line), interquartile distance (box), 3 × interquartile distance or extremes (whiskers), and outliers (spots) are indicated. Figure 7—figure supplement 1 shows results of individual centromeres. Asterisk indicates statistically significant difference (t test; p<0.05); NS indicates no significant difference. (G) Representative images of CENP-A antibody staining in indicated cell types. Images of RPE cells are shown as independent reference. Primary fibrobl. indicates primary human foreskin fibroblasts. (H) Quantification of G. Mean ± SEM for n = 3–4 independent experiments is shown. Left y-axis represents centromeric CENP-A levels normalized to RPE cells; right y-axis shows number of CENP-A molecules per centromere (CEN). (I) Combined results from A–H allow for the determination of CENP-A copy numbers on individual chromosomes as indicated. (J) Statistical map of the distribution of 216 CENP-A nucleosomes on the NeoCEN-4 at three different scales. The top 216 peaks are indicated in blue. Y-axis indicates the probability of CENP-A occupancy for each nucleosome. (K) Histogram of the CENP-A nucleosome occupancy. Inset shows the distribution of 216 neocentric CENP-A nucleosomes on the 10% highest occupancy peaks (green) and 90% lowest occupancy peaks (red).

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Figure 7—figure supplement 1. Measurements of individual centromeres for graphs in Figure 7A–F.
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Figure 8. A quantitative view of human centromeric chromatin. (A) Distribution of CENP-A. Estimated ratio of CENP-A (red) to H3 (gray) at the centromere and on non-centromeric loci (genome) in interphase cells. Estimations are calculated assuming 2 CENP-A molecules per nucleosome (Sekulic et al., 2010; Tachiwana et al., 2011; Bassett et al., 2012; Hasson et al., 2013; Padeganeh et al., 2013), an average nucleosome positioning distance of 200 base pairs, an average centromere size of $2.5 \times 10^6$ base pairs (Sullivan et al., 1996; Lee et al., 1997) of which approximately 40% (1 Mbp) contains CENP-A (Sullivan et al., 2011), a diploid genome size of $6 \times 10^9$ base pairs, 200 CENP-A nucleosomes per centromere, and $2.5 \times 10^4$ CENP-A nucleosomes outside of centromeres ($9.1 \times 10^4$ CENP-A molecules per cell [Figure 2F], of which 74% is in chromatin [Figure 4B] and 0.44% in each centromere [Figure 2B]). The fraction of CENP-A on centromeres, non-centromeric chromatin, and unincorporated CENP-A are indicated in green, blue, and black, respectively. CENP-A nucleosomes are represented as though evenly spread throughout the centromeric domain. Alternatively, they could be distributed into one or more clusters within this domain. (B) Mitotic organization of centromeric chromatin. 200 nucleosomes are redistributed to 100 nucleosomes per centromere on replicated sister chromatids (Jansen et al., 2007; Bodor et al., 2013). The exact CENP-A copy number at the centromere depends on the available total pool (mass-action). Excess CENP-A could either lead to an increased CENP-A domain or lead to a higher density of CENP-A within a domain of fixed size. This pool forms an excess to recruit downstream centromere and kinetochore complexes and ultimately provides microtubule binding sites for $\sim 17$ kinetochore microtubules (McEwen et al., 2001). To avoid mitotic errors, a critical amount of CENP-A is required (dashed lines). (C) Graph representing the chance of at least one Figure 8. Continued on next page
Figure 8. Continued

chromosome in a cell (with 46 chromosomes) reaching critically low levels of CENP-A by random segregation of
pre-existing CENP-A nucleosomes. Calculations were performed for varying levels of critical nucleosome numbers
at a fixed steady state of 200 (left), or by varying the steady state number at a fixed critical level of 22 (right). Red
bars represent identical calculations.

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