Inventory of Supplemental Information

Supplemental Figure Legends

Supplemental Figure 1, related to Figures 1-4. FACS analysis and CENP-A IP scheme for synchronized HeLa and HEK cells. **(A)** Cells were synchronized using double thymidine block followed by harvesting at various points of the cell cycle. FACS analysis confirms enrichment of cell-cycle specific stages. Solid red line indicates level of peak propidium iodide (PI) stain from previous time point, and dotted red line indicates current PI staining levels for comparison. Nuclear content (2N and 4N) designations are placed below to indicate stage of cell cycle. **(B)** Schematic of AFM and CENP-A ChIP and **(C)** CENP-A IP and LC-MS/MS.

Supplemental Figure 2, related to Figure 1. Native chromatin IP of CENP-A nucleosomes demonstrate they contain alpha satellite DNA and HJURP IP demonstrates change in binding to CENP-A dependent on cell cycle. (A) SDS-PAGE analysis of input chromatin showing equivalence of core histones in extracted chromatin fibers, and sub-fraction of CENP-A in WB panel. (**B)** Quantitative enrichment of CENP-A during IP procedures was calculated from a comparison of CENP-A signal on WB across 1% input, 10% unbound and 10% CENP-A-IP lanes. Image J was used to quantify the LiCor Odyssey signal intensity showing a 3-4 fold enrichment in the IP. **(C)** PCR amplification of centromere-specific alpha satellite DNA from equal amounts of CENP-A IP from G1, S and G2 phases confirm that CENP-A nucleosomes contain centromeric DNA. Negative control is the No Taq polymerase lane. **(D)** HJURP IP demonstrates that although HJURP is always associated with chromatin (upper panel), CENP-A is enriched in G1 and G2 phases, but not at S phase (WB panel, CENP-A). **(E)** Chromatin fiber

immuno-fluorescence analysis demonstrates that HJURP is released from CENP-A chromatin at S phase, but returns at G2 and G1. CENP-A red, HJURP green, DNA (DAPI) blue. Scale bar, 1μM.

Supplemental Figure 3, related to Figure 2. CENP-A nucleosome have consistent diameters at all points of the cell cycle and H3 IP nucleosomes have octameric dimensions. (A) Images of CENP-A arrays obtained from various points of the cell cycle. Scale bar 10nm. **(B)** Diameter of CENP-A nucleosome is unchanged throughout the cell cycle (Table 1). **(C)** Control H3 nucleosomal IPs from G1 and G2 cells demonstrate they have octameric dimensions (overlay of H3 IP in green and bulk chromatin in gray).

Supplemental Figure 4, related to Figure 2. CENP-A nucleosomes contain less DNA at G2/M phase compared to G1/S and S phase. **(A)** G2/M CENP-A nucleosomes (inset, 3D, scale bar 10nm) have tetrameric dimensions (line section profile, bottom) and after protease treatment, release far less DNA (inset, Scale bar 100nm), **(B)** compared to multiples of the canonical 150-250bp repeat released by S phase CENP-A nucleosomes. Scale bar 100nm. N refers to mono, di and trinucleosomes. Red line indicates octameric shift.

Mean values (bp) and counts (n) for

G2/M phase CENP-A: N1=115 ± 30, N2= 221± 33 (n=145).

G1/S phase CENP-A: N1=156 ± 27, N2*= 227 ± 24, N2=315±43 (n=194).

S phase CENP-A: N2= 285±76, N3= 454 ± 30, N4= 588 ± 52 (n=207).

(C) GFP-CENP-A Immuno-precipitation shows enrichment of native CENP-A at S-phase but not at G1/S. Western blot using an anti-CENP-A antibody reveals relative proportion of GFP-CENP-A and native CENP-A within the IP. Quantification of the results demonstrate that native CENP-A is 7-fold enriched in S-phase GFP-CENP-A IP over G1/S CENP-A IP, suggesting CENP-A: CENP-A interactions are more robust in S phase. Anti-CENP-A Control Western blot performed using just agarose beads below. Asterisk (*) is cross-reacting agarose bead band.

Supplemental Figure 5, related to Figure 3. Cell-cycle dependent analysis of FRET efficiency between H3.1 pairs vs. CENP-A pairs demonstrates CENP-A has unique dynamics. (A) Localization pattern of CENP-F, visualized by immuno-fluorescence, of CENP-A-EGFP of the CENP-F stained cells (2^{nd} row), and of PCNA, visualized by immuno-fluorescence. CENP-F is absent during G1, becomes detectable in early S-phase and is diffuse during late S-phase and G2. During mitosis, it forms distinct foci at kinetochores co-localizing with CENP-A. PCNA staining is diffuse during G2, mitosis, and G1. It forms distinct foci during S-phase allowing the identification of early, mid, and late S-phase. Bars: 5 µm. **(B)** FRET between N- and C- terminal tagged H3.1-EGFP and H3.1-mCherry pairs is constant during the cell cycle. Red bars, significant FRET ($p < 0.001$) between EGFP-H3.1 and mCherry-H3.1; green bars, significant FRET ($p < 0.001$) between. Significant FRET was obtained in each analyzed sample (see also Supp Table 1). **(C)** FRET between the N-termini of CENP-A show FRET at G1 and G1/S phase, but reduced FRET during the rest of the cell cycle. **(D)** FRET analysis of C-terminally tagged CENP-A/CENP-A in isolated nuclei of G1/S cells, showing a comparison of FRET

values obtained from untreated and MNase-treated nuclei. The untreated nuclei showed significant FRET (black bar; $p<0.001$) whereas the MNase treated nuclei showed not FRET (grey bar). (Table S presents detailed results). **(E)** The disappearance of FRET between CENP-A/CENP-A during S phase is not due to replication of centromeres. FRET values measured 4 hr after release from double thymidine block remain constant when cells were released in the presence of aphidicolin, however FRET disappears when aphidicolin is added only 1 hr after release into S phase. Black bars, significant FRET ($p<0.001$); grey bars no FRET.

Supplemental Figure 6, related to Figure 4. CENP-A and H4 have unique modifications at G1/S. (A) Cells were synchronized (refer to Supplemental Figure 1, FACS Analysis), extracted, and chromatin was bound to hydroxylapatite (refer to Supplementary Figure 2B, purification scheme). Released histones were immuno-precipitated using CENP-A antibody, ran on SDS-PAGE gel, bands of interest were isolated and subjected to LC-MS/MS for discovery of protein modifications. (**B)** CENP-A was purified (as described in Materials and Methods) from HeLa and human embryonic kidney (HEK) cells in G1/S phase, separated from contaminants on SDS-PAGE, and subjected to in-gel tryptic digestion. The position of the monoisotopic ion ($[M+2H^+]$, m/z 714.90) for CENP-A peptide "VTLFPK(acetyl)DVQLAR" is in the scan # 8089, at the beginning of the peptide elution at 35.52 min. of the chromatogram (indicated with a red diamond). The inset depicts the magnified region around the ion (top) and the same scale zoom of the later scan, at the chromatographic elution peak (eluting at 35.60 min of the chromatogram) of the peptide (bottom). The ion chosen for

fragmentation is the next heavier isotopic peak (m/z 715.42), indicated with a blue diamond. MS/MS spectra resulting from the CID fragmentation of the m/z 715.42 ion from Figure 4a. **(C)** Depicts the fragmentation of the non-acetylated, methionine-oxidized tryptic peptide of H4 KTVTAM(oxi)DVVYALK, as doubly charged, monoisotopic ion, m/z 727.90 (+0.22 ppm difference from the theoretical m/z) from scan 4448, eluting at 28.59 min. of the chromatogram.

Supplemental Figure 7. CENP-A Dynamics Across the Cell Cycle. Summarizes CENP-A changes over the cell cycle related to structure, kinetochore protein and chaperone binding, and histone modifications observed in this study.

Supplementary Table 1, related to Figure 3. FRET measurements demonstrate that CENP-A chromatin is highly dynamic over the cell cycle

		Bleached kinetochores		Unbleached kinetochores			
Cell-cycle Phase	Synchronization Protocol	N	E_{FRET}	N	E_{VAR}	FRET Value	Significant FRET
CENP-A-EGFP and CENP-A-mCherry							
Late G1/S	TTB	63	5.9 %	63	0.4 %	5.5 %	+
S (2 hr)	TTB	46	1.8 %	46	1.1 %	0.7 %	-
S (4 hr)	TTB	52	2.5 %	52	1.8 %	0.9 %	-
S (6 hr)	TTB	40	3.6 %	40	3.4 %	0.2 %	-
G2	TTB	43	3.9 %	40	3.0 %	0.9 %	-
Mitosis	TTB	62	2.0 %	62	1.9 %	0.1 %	-
C	TB-RO 3306	45	2.2 %	45	1.8 %	0.4 %	-
G1 (3 hr)	TB-RO 3306	49	4.1 %	49	-0.4 %	4.5 %	+

G1 (5 hr)	TB-RO 3306	67	4.8 %	67	0.3 %	4.3 %	+
G1 (7 hr)	TB-RO 3306	83	4.1 %	83	0.0 %	4.1 %	+
G1 (9 hr)	TB-RO 3306	65	4.1 %	65	-0.3 %	4.4 %	+
G1 (11 hr)	TB-RO 3306	65	5.1 %	65	0.8 %	4.3 %	+
G1 (13 hr)	TB-RO 3306	68	5.5 %	68	0.3 %	5.2 %	+
G1 (15.5 hr)	TB-RO 3306	67	4.8 %	67	0.4 %	4.4 %	+
S (17 hr)	TB-RO 3306	61	1.6 %	61	0.6 %	1.0 %	-
EGFP-CENP-A and mCherry-CENP-A							
Late G1/S	TTB	36	11.7 %	36	1.9 %	9.8 %	+
S (2 hr)	TTB	73	7.3 %	73	4.8 %	2.5 %	-
S (4 hr)	TTB	49	4.0 %	49	2.6 %	1.4 %	-
S (6 hr)	TTB	45	6.9 %	45	4.9 %	2.0 %	-
G2	TTB	28	1.9 %	28	1.1 %	0.8 %	-
Mitosis	TTB	51	3.7 %	51	3.3 %	0.4 %	-
C	TTB	20	4.8 %	20	4.6 %	0.2 %	-
EGFP-H3.1 and mCherry-H3.1							
Late G1/S	TTB	45	0.2 %	45	-2.2 %	2,4 %	+
S (4 hr)	TTB	35	0.7 %	35	-2.6 %	3.3 %	+
S (8 hr)	TTB	35	0.9 %	35	-2.7 %	3.6 %	+
H3.1-EGFP and H3.1-mCherry							
Late G1/S	TTB	40	3.8 %	40	0.4 %	3.4 %	+
S (4 hr)	TTB	40	2.0 %	40	-1.2 %	3.2 %	+
S (8 hr)	TTB	50	3.6 %	50	0.2 %	3.4 %	+

TTB = double Thymidine block; TB-RO 3066 = single Thymidine block in combination with RO 3306. N=number of kinetochores measured.

Supplementary Table 2, related to Figure 3. FRET values of CENP-A result from

chromatin fiber interactions, and are not influenced directly by centromere replication

			Bleached kinetochores		Unbleached kinetochores			
Cell-cycle Phase	**Synchronization method**	**Treat-ment**	**N**	**E_{FRET}**	**N**	**E_{VAR}**	**FRET %**	**Sign. FRET**
								(p<0.001)
CENP-A-EGFP and CENP-A-mCherry in isolated nuclei								
Late G1/S	TTB	-	52	5.4 %	52	1.0 %	4.4 %	+
Late G1/S	TTB	MNase	49	2.5 %	49	1.4 %	1.1 %	-
CENP-A-EGFP and CENP-A-mCherry (aphidicolin treatment)								
Late G1/S	TTB	-	58	6.7 %	58	2,5 %	4.2 %	+
S (4 hr)	TTB	Aphid. 0 hr after release TBBrelease	65	4.6 %	65	0.5 %	4.1 %	+
S (4 hr)	TTB	Aphid. 1 hr after release	58	4.0 %	58	3.1 %	0.9 %	-
S (4 hr)	TTB	Aphid. 2 hr after release	62	0.6 %	62	0.4 %	0.2 %	-

TTB = double thymidine-block; Aphid = Aphidocolin.

Supplemental Experimental Procedures

GFP-CENP-A Study

pEGFP-C1-CENP-A construct was transfected into HeLa cells and stables were generated by supplementing DMEM media with 1,500 mg/mL geneticin After selection, stable HeLa cells expressing pEGFP-C1-CENP-A were synchronized with a double thymidine block to G1/S and S-phases of the cell cycle, nuclei digested for 8 min with

MNase and extracted overnight in 0.5X PBS/5 mM EGTA, and IP'ed using mouse monoclonal GFP (B-2, Santa Cruz Cat # sc-9996) antibody. Both GFP-CENP-A and native CENP-A were detected by Western with rabbit αCENP-A antibody (Millipore Cat # 07-574), imaged with LiCor Odyssey and band intensities were quantified by LiCor's Image Studio.

Plasmids used in FRET study

For the expression of human CENP-A fused with its N-terminus to EGFP we used pEGFP-C1-CENP-A and for the expression of human CENP-A fused with its N-terminus to mCherry, the required plasmid was constructed from the vector pCerulean-C1-CENP-A (Orthaus et al., 2008). This vector was digested with AgeI-BsrGI. Into the resulting purified 4.433 bp fragment we ligated a 713 bp AgeI-BsrGI fragment resulting from the vector pmCherry-C1 (Orthaus et al., 2009). All clones were verified by sequencing (MWG Biotech, Ebersberg, Germany). For the fusion of fluorescent proteins to the C-terminus of CENP-A, we amplified full length CENP-A by PCR (Expand high fidelity^{PLUS} PCR System, Roche, Penzberg, Germany) applying forward primer 5´-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGAAAACCTGTATTTTCAGGGC GCCACCATGGGCCCGCGCCGCCGGA -3´ and reverse primer 5´-GGGGACCACTTTGTACAAGAAAGCTGGGTGCCGAGTCCCTCCTCAAGGCC -3. The CENP-A harbouring linear PCR fragment was transferred into vector pDONR221 by BP recombination reaction (Invitrogen, Carlsbad, CA, USA). After verification by sequencing, the gene was cloned by LR recombination reactions into modified pEGFP-N and mcherry-N based destination vectors. As the result we obtained expression vectors

carrying the gene coding for CENP-A fused to the N-termini of EGFP and mcherry. In the constructs, the linker sequence between CENP-A and the fluorescent protein is TQLSCTKWLDPPVAT. Full length protein expression of the fusion constructs was confirmed by Western Blots. For transfection, HEp-2 or HeLa cells were harvested at a confluence of 70 – 80%. Aliquots of 10^6 cells were centrifuged for 10 min at 1.600 rpm. The medium was removed and cells were suspended in 100 µl Nucleofector solution V (Amaxa Inc., Walkersville, USA) containing 1 µg pEGFP-C1-CENP-A and pmCh-C1-CENP-A, or pH-CENP-A-G and pH-CENP-A-Ch, or pEGFP-H3.1 and pmCh-H3.1, or pH3.1-EGFP and pH3.1-mCh, each, followed by electroporation with the Nucleofector (Amaxa Inc., Walkersville, USA) and subsequently seeded for expansion.

Immuno-fluorescence

For indirect immuno-fluorescence detection, HEp-2 cells were fixed with 4% PFA in PBS for 15 minutes or with methanol for 5 minutes, permeabilised with 0.5% Triton X-100 in PBS for 10 minutes and blocked with 3% bovine serum albumin (BSA) in 1X PBS. Primary rabbit anti CENP-F antibodies (Novus Biologicals, Littleton, USA) were diluted 1:750 in 1X PBS. They were detected with fluorescently labelled goat anti-rabbit antibodies (Cy5 anti-rabbit, Dianova, Hamburg, Germany) at a dilution of 1:200. Primary mouse anti PCNA antibodies (Invitrogen, Carlsbad, CA, USA) were diluted 1:200 in 1X PBS. They were detected with fluorescently labelled goat anti-mouse antibodies (Cy5 anti-rabbit, Dianova, Hamburg, Germany) at a dilution of 1:200. Fluorophores were stimulated with 633 nm Helium-Neon-Laser line with low intensity and signals were detected via a 650 nm long path filter.

Acceptor photobleaching based FRET measurements (AB-FRET)

FRET measurements were performed with fixed cells. FRET performed with the same protein pairs in living cells and in fixed cells were compared, and no detectable difference was observed (data not shown). When FRET occurs, both the intensity and lifetime of the donor fluorescence decrease while the intensity of the acceptor emission increases. Such changes can be exploited to measure the efficiency of energy transfer between the donor and the acceptor (Chen et al., 2003; Elder et al., 2009; Jares-Erijman and Jovin, 2003; Nagy et al., 1998). Here, the FRET pair EGFP-mCherry was used. EGFP fluorescence was excited with the Argon 488 nm laser line and analysed using the Meta detector (ChS1+ChS2: 505-550 nm). mCherry fluorescence was excited with the 561 nm laser line (DPSS 561-10) and detected in one of the confocal channels using a 575-615 nm band-pass filter. To minimize cross talk between the channels, each image was collected separately in the multi-track-mode, i.e. both fluorophores were excited and recorded specifically and separately. By eye inspection we searched for cells moderately expressing both fusion proteins with comparable expression levels. Single optical sections were selected by scanning the sample in the z-axis for optimal fluorescence signals with 8-fold magnification. Acceptor photobleaching was achieved by scanning a region of interest (ROI) including up to 5 centromeres of a nucleus 50 times (scans at 1.6 µs pixel time) using the 561 nm laser line at 100% intensity. Bleaching times per pixel were identical for each experiment, however, total bleaching times varied depending on the size of the bleached ROIs. Four donor and acceptor fluorescence images were taken before and four images were taken after the acceptor photobleaching procedure to assess

changes in donor and acceptor fluorescence. To minimize the effect of photobleaching of the donor during the imaging process, the image acquisition was performed at low laser intensities. To compare the time course of different experiments, (i) donor intensities in the ROI were averaged and normalized to the intensity measured at the first time point after photobleaching and (ii) acceptor intensities in the ROI were averaged and normalized to the mean intensity measured at time points two – four before photobleaching.

The FRET efficiency was calculated according to $E_{FRET}=1-(I_{DA}/I_D)$ with I_{DA} and I_D the donor fluorescence in the presence and absence of the acceptor, respectively. I_{DA} was obtained by averaging the donor intensities of pre-bleach images two - four in the presence of a photo-chemically intact acceptor. I_D was determined by measuring the donor fluorescence intensity in the first image obtained after the acceptor has been destroyed by photobleaching. At kinetochores of the unbleached location, the donor fluorescence change was calculated according to $E_{var}=1-(I_{DAbefore}/I_{DAafter})$ with the donor fluorescence intensities $I_{DAbefore}$ and $I_{DAafter}$ before and after photobleaching, repectively. The "variation efficiency" E_{var} can directly be compared to the FRET efficiency E_{FRET}. From each cell we analyzed the same number of bleached and unbleached kinetochores. The E values E_{FRET} and E_{var} were grouped into boxes of 4% and the numbers of cases (y-axis) were plotted versus E values (x-axis) at the x-axis values of the middle of the box (0-4%: middle value 2%, 4-8%: middle value 6%, etc.) resulting in distribution diagrams of both, E_{FRET} and E_{var} (see Fig. Xa and Xb). E_{var} indicates the donor fluorescence intensity variation during the experiment in the same cell due to background effects. The

E_{FRET} and E_{VAR} values were compared by a paired t-test ($\alpha = 0.05$). The difference between the means of E_{FRET} and E_{VAR} is a measure for the FRET-value, which was interpreted to have occurred when the mean of E_{FRET} is clearly larger than the mean of E_{var} and in addition the paired t-test revealed a statistically significant difference between the two input groups with a p-value below 0.001. A p-value ≥ 0.001 was interpreted as an indication for insignificant FRET.

If the orientation of the fluorophore dipole moment of the acceptor relative to that of the donor were known, or at least one of them would rotate freely faster than nsec, a more detailed distance between donor and acceptor could be deduced from the measured E_{FRET} values. In our experiments however, this information is not available to us. We therefore do not deduce defined distance values but interpret the appearance of FRET as an indication that donor and acceptor fluorophores are close to one another within 10 nm.

Aphidicolin treatment

To analyse the influence of replication of the centromeric regions on FRET between CENP-A/CENP-A, double transfected Hep2 cells (CENP-A-EGFP and CENP-A mCherry) were treated with double thymidine block. Aphidicolin was added (final concentration 5 µg/ml) simultaneously with the release (0 hr), respectively 1 hr or 2 hr after release. FRET was measured 4 hr after release. As control FRET was measured in cells, which were not treated with aphidicolin, immediately before release and 4 hr after release.

Preparation of nuclei and MNase treatment

CENP-A/CENP-A double transfected cells (CENP-A-EGFP and CENP-A-mCherry) were treated with a double thymidine block and harvested at the time point of release. After washing twice in PBS cells were re-suspended in buffer N (Tris-HCl 15 mM, pH 7.5, KCl 60 mM, NaCl 15 mM, $MgCl_2$ 5 mM, $CaCl_2$ 1 mM, DTT 1 mM, Na-Vanadate 2 mM, sucrose 250 mM). Subsequently NP40 was added to a final concentration of 0.3 % and cells were incubated for 5 min on ice. Nuclei were washed twice in buffer N. For MNase treatment 50 U were added to 250 µl suspension and incubated for 10 min at 37°C. For microscopic analysis untreated and treated nuclei were placed on polylysine coated microscope slides and fixed with 4 % paraformaldehyde in PBS.

Supplemental References

Chen, Y., Mills, J.D., and Periasamy, A. (2003). Protein localization in living cells and tissues using FRET and FLIM. Differentiation *71*, 528-541.

Elder, A.D., Kaminski, C.F., and Frank, J.H. (2009). phi2FLIM: a technique for alias-free frequency domain fluorescence lifetime imaging. Opt Express *17*, 23181-23203.

Jares-Erijman, E.A., and Jovin, T.M. (2003). FRET imaging. Nat Biotechnol *21*, 1387-1395.

Nagy, P., Bene, L., Balazs, M., Hyun, W.C., Lockett, S.J., Chiang, N.Y., Waldman, F., Feuerstein, B.G., Damjanovich, S., and Szollosi, J. (1998). EGF-induced redistribution of erbB2 on breast tumor cells: flow and image cytometric energy transfer measurements. Cytometry *32*, 120-131.