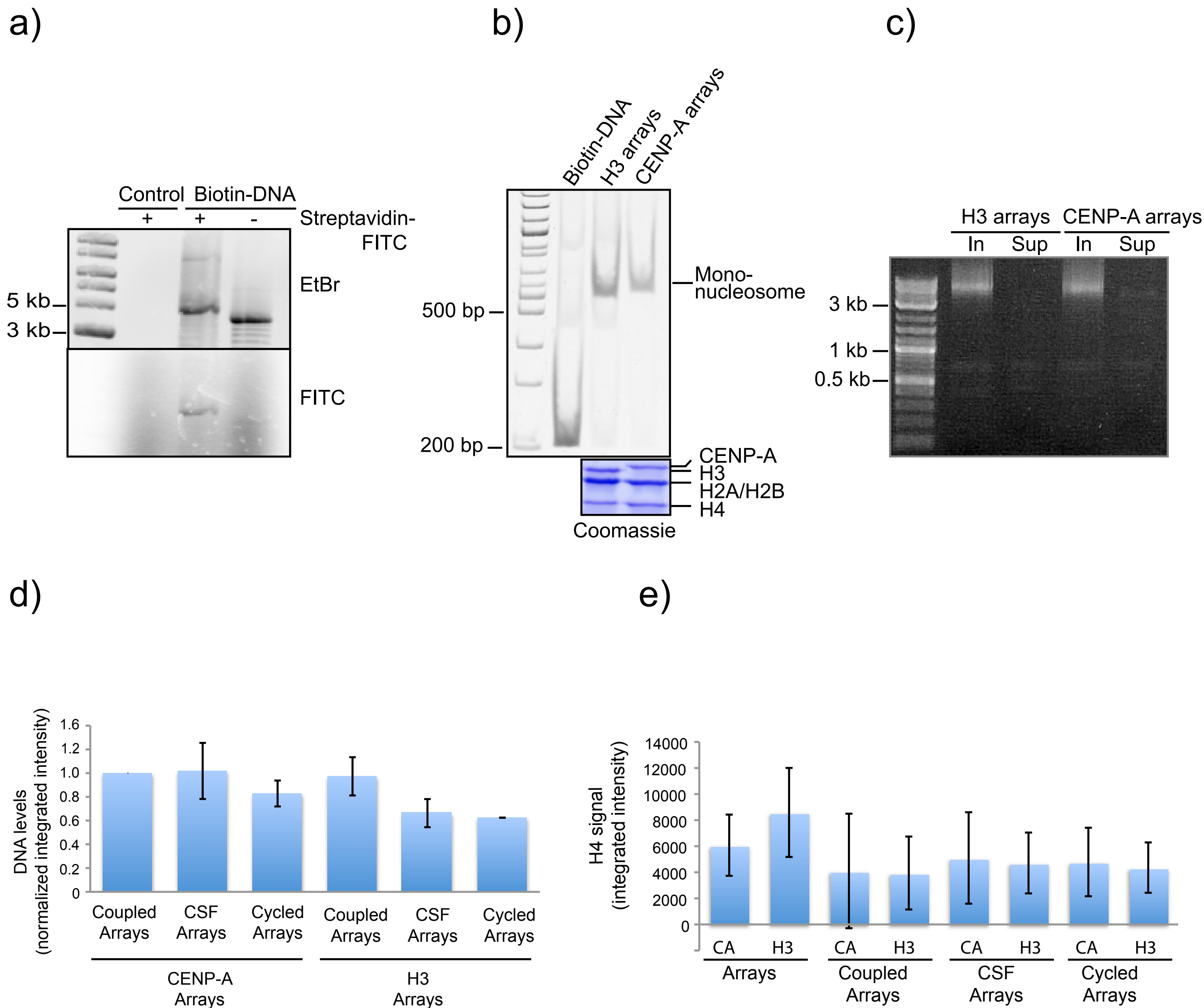


Figure S1

Reconstituted chromatin is efficiently coupled to Streptavidin-coated magnetic beads and stably maintained in *Xenopus* egg extracts.
(a) Biotinylated array DNA (Biotin-DNA) or a control (no DNA) was incubated with (+) or without (-) Streptavidin-FITC and subsequently separated by agarose gel electrophoreses. The DNA was visualized by Ethidium Bromide (EtBr) stain. Bound Streptavidin-FITC (FITC) caused a gel mobility-shift and could be detected by fluorescence imaging (lower panel). (b) CENP-A and H3 chromatin assembled with equal efficiency, at >90% saturation of the positioning sites and with equal histone protein stoichiometry. Control DNA (first lane), and assembled H3 and CENP-A chromatin (second and third lane) were resolved on a native gel after AvaI digestion to generate ~ 200 bp DNA fragments and/or H3 and CENP-A mononucleosomes (~ 700 bp). The lower panel shows the stochiometry of histones in undigested chromatin arrays by Coomassie stain. (c) CENP-A and H3 chromatin arrays on biotinylated DNA bound equally efficiently to Streptavidin-coated magnetic beads. The input chromatin array DNA (500 ng) and a corresponding amount of the supernatant (Sup) after chromatin coupling to Streptavidin coated magnetic beads were separated by agarose gel electrophoresis and stained with EtBr. (d) Quantification of DNA levels by fluorescence microscopy of chromatin beads after coupling and after incubation in CSF and cycled egg extracts. The levels are rescaled so that DNA levels coupled to CENP-A arrays (Coupled Arrays) are normalized to 1. Error bars represent SEM, n = 2 (e) Quantification of H4 levels using western blot analysis. Error bars represent SEM, n=3

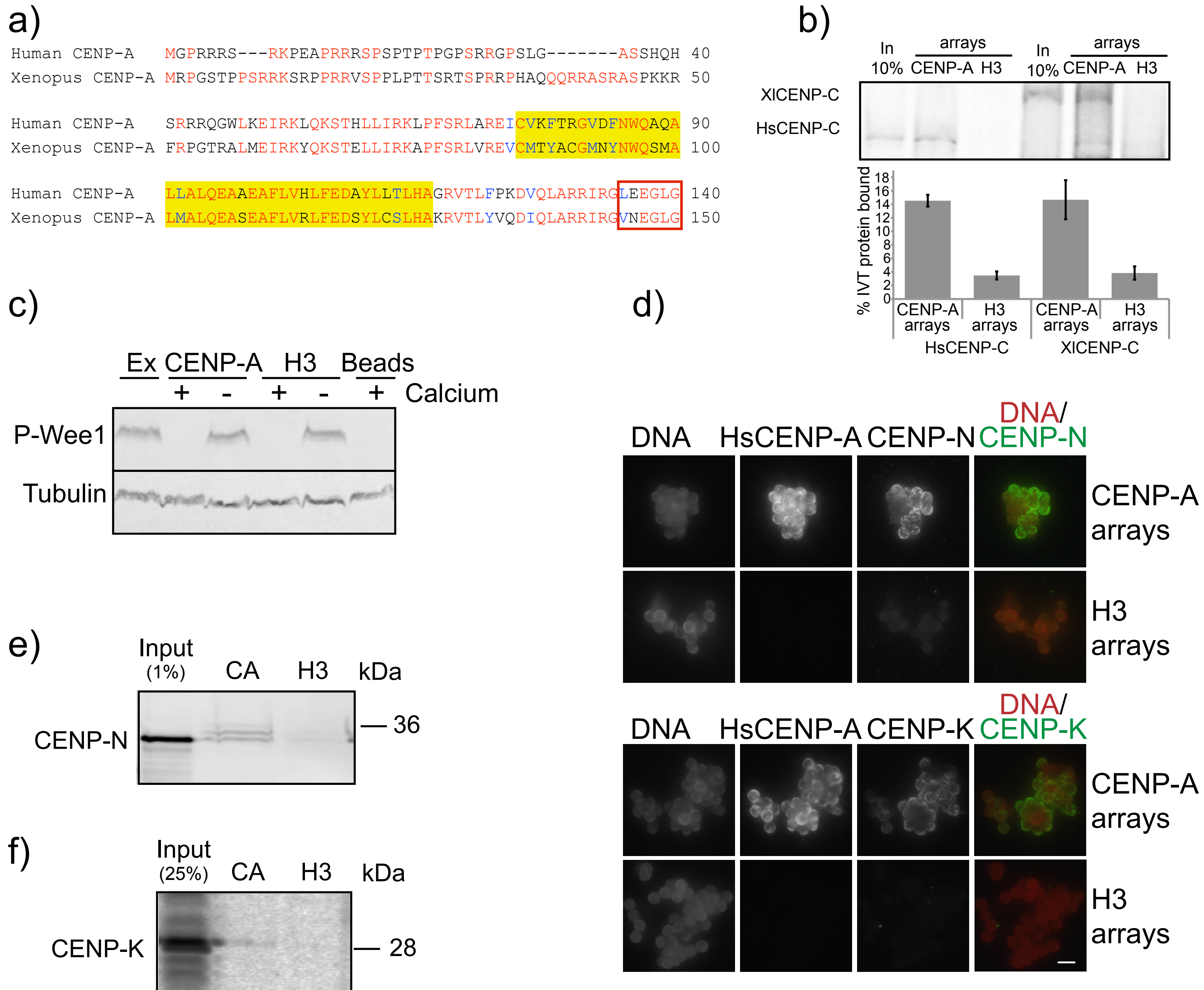


Figure S2

CENP-A is conserved and specifically binds to CENP-C, CENP-N and CENP-K. (a) Protein sequence alignment showing high conservation between human CENP-A and *Xenopus* CENP-A. The CENP-A CATD domain (AA75 – AA114 for Human CENP-A) is shown in yellow and the conserved C-terminus (AA135 – AA140 for Human CENP-A) is highlighted in a red box. (b) *In vitro* binding of human CENP-C (HsCENP-C) and *Xenopus* CENP-C (XlCENP-C) to CENP-A chromatin arrays in comparison to H3 chromatin arrays. HsCENP-C and XlCENP-C were *in vitro* translated in the presence of 35S-methionine, incubated with CENP-A and H3 chromatin (60 nM nucleosomes) and the protein bound to the arrays was separated by SDS-PAGE and detected by autoradiography (upper panel). Quantification of CENP-C binding to chromatin arrays is shown as % bound, normalized to the input (In) set at 100%. Error bars represent SEM, n = 3 (lower panel). (c) Western blot for phoshorylated Wee1 (P-Wee1) as an indicator of the cell cycle stage of the extract. P-Wee1 signal is high in CSF extracts (- Calcium) and low in interphase extracts (+ Calcium). Tubulin is shown as a loading control. (d) Representative immunofluoresecence images of CENP-N (top) and CENP-K (bottom) binding to CENP-A and H3 chromatin arrays in CSF extracts. The left panel shows the DNA, the adjacent panel to the right HsCENP-A, and the third panel shows each centromere protein. A merge image of the DNA (red) and the each centromere protein (green) is shown (DNA/Protein; right panel). Scale bar, 5 μm (e) Myc tagged CENP-N was *in vitro* translated and incubated for 60 min, together with either CENP-A or H3 chromatin beads, in CSF *Xenopus* egg extracts. Bound Myc-CENP-N was separated by SDS-PAGE and visualized using western blot analysis and α-Myc-antibodies. (f) CENP-K was *in vitro* translated and radioactively labeled using 35S-methionine, before incubation with either CENP-A or H3 chromatin in *Xenopus* egg extracts. The reactions were separated by SDS-PAGE and visualized by autoradiography.

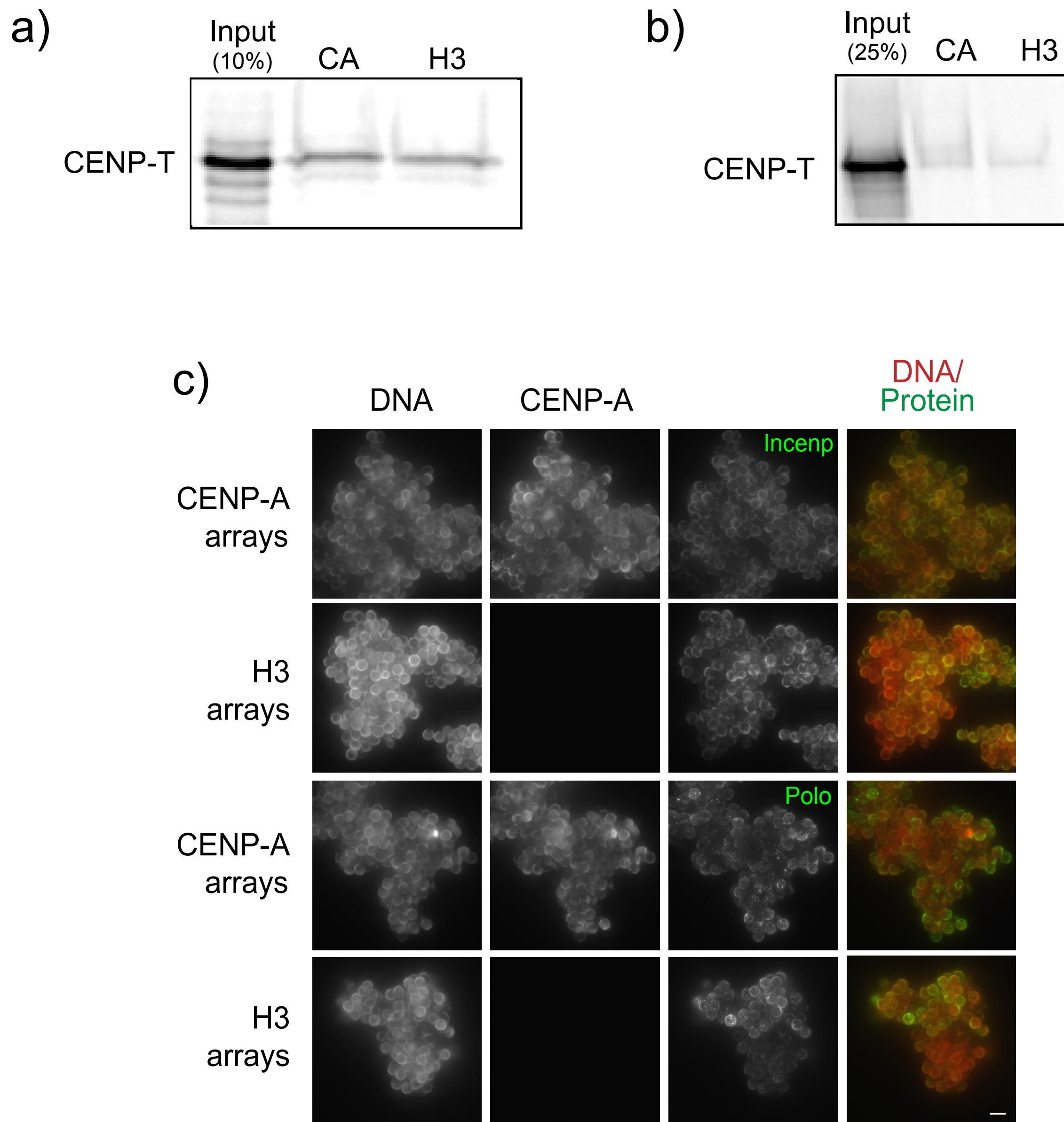


Figure S3

CENP-T, Incenp and Polo kinase bind to CENP-A and H3 chromatin. (a) *In vitro* binding of CENP-T to CENP-A and H3 chromatin beads. CENP-T was translated in the presence of 35S-methionine and incubated with CENP-A and H3 chromatin. CENP-T bound to the arrays was separated by SDS-PAGE and detected by autoradiography. (b) *In vitro* translated and radioactively labeled CENP-T was incubated with either CENP-A or H3 chromatin in *Xenopus* CSF extract before bound CENP-T was separated by SDS-PAGE and analyzed by autoradiography (c) Immunofluorescence analysis showing Incenp and Polo kinase binding to CENP-A and H3 arrays in *Xenopus* CSF extract.

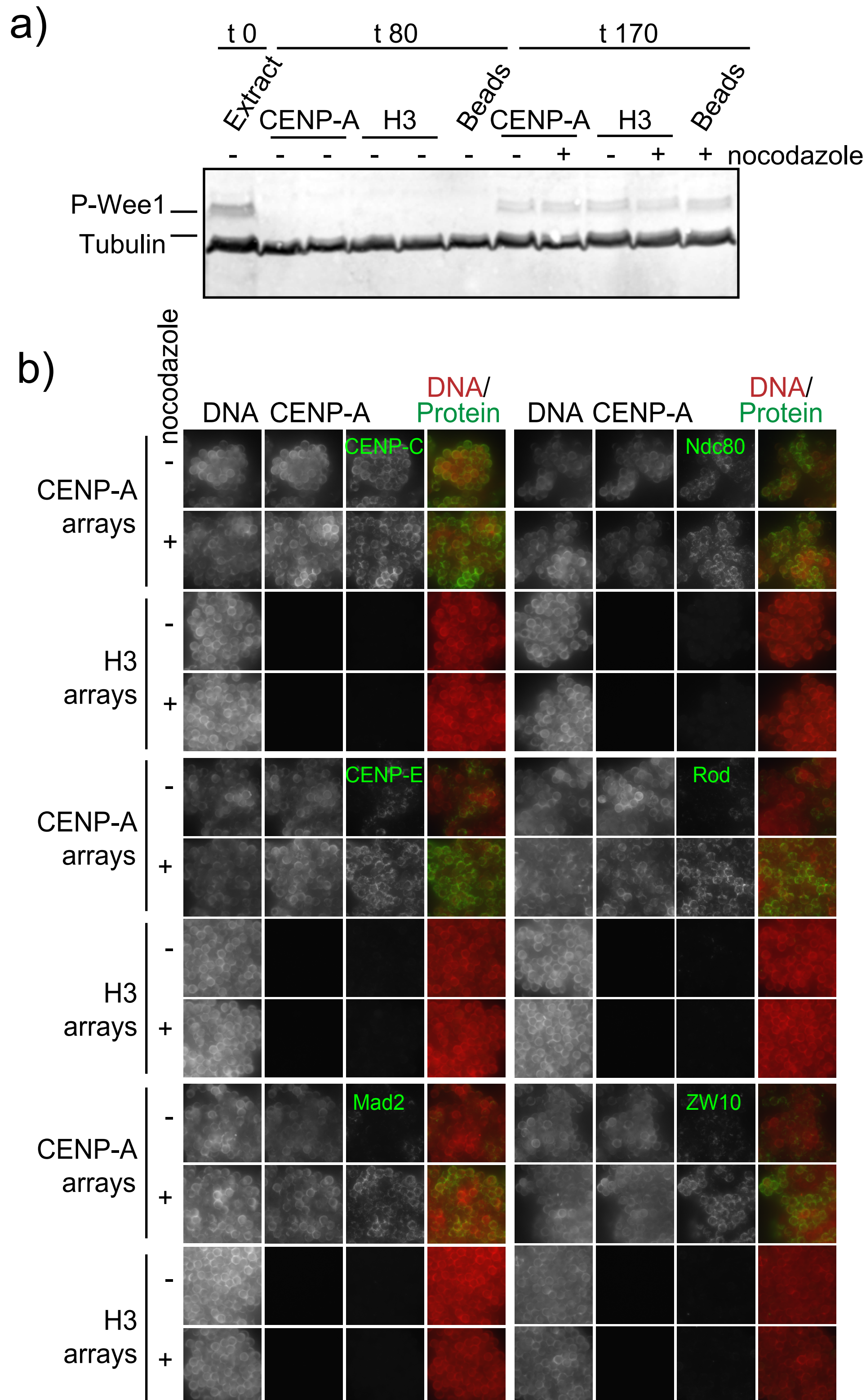


Figure S4

Kinetochore proteins are specifically recruited to CENP-A chromatin (a) A western blot of phospho-Wee1 staining to monitor the mitotic state during extract cycling. Microtubule depolymerization (+/- nocodazole) does not affect the reentry of the extract into mitosis. Tubulin levels are shown as a western blot loading control. (b) Representative immunofluorescence images showing the CENP-C, Ndc80, CENP-E, Mad2, Rod and ZW10 recruitment to CENP-A and H3 chromatin arrays with and without (+/-) nocodazole. The left panel shows the DNA. The next two panels to the right show HsCENP-A and the different proteins analyzed: CENP-C, Ndc80, CENP-E, Mad2, Rod and ZW10, respectively. A merge of the DNA (red) and CENP-C, Ndc80, CENP-E, Mad2, Rod or ZW10 (green) channels is shown in the right panel (DNA/Protein). Scale bar, 5 µm

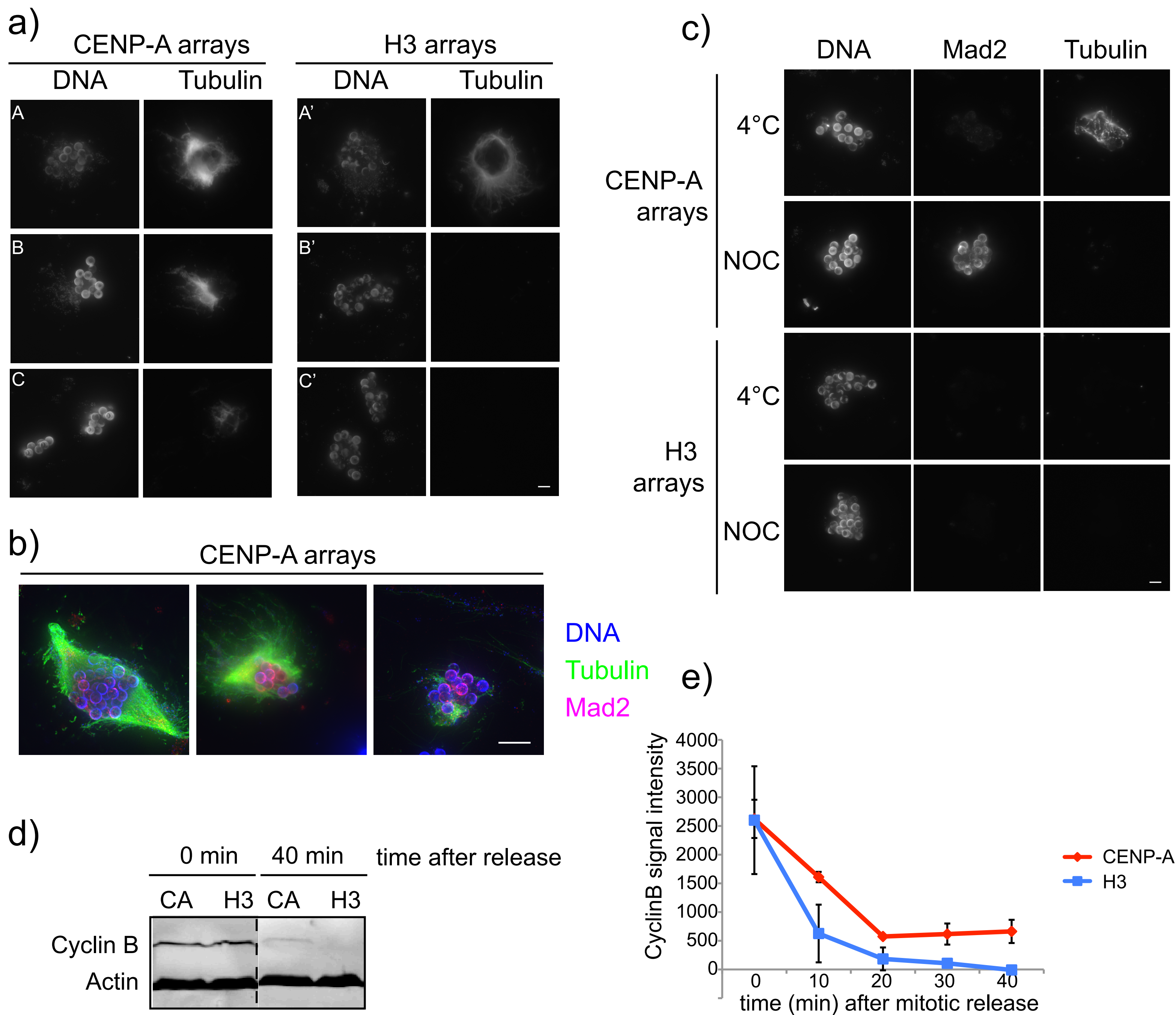


Figure S5

Microtubules specifically associate with CENP-A chromatin and are stabilized at 4°C. (a) Representative images of phenotypes observed for tubulin association with CENP-A and H3 chromatin arrays used for quantification in Figure 3b. Chromatin arrays were incubated for 90 min in CSF egg extracts at 20°C followed by fixation, sedimentation and localization of DNA and tubulin. Microtubules polymerize around groups of CENP-A and H3 chromatin beads (A, right panel and A', right panel). Most H3 containing beads do not associate with microtubule structures (B' and C', right panel) while the majority of CENP-A containing beads have associated tubulin (B and C, right panel). Scale bar, 5μm (b) Representative images of microtubule structures associated with CENP-A chromatin arrays. Tubulin is shown in green, DNA in blue and the spindle checkpoint protein Mad2 in magenta. Bipolar spindles (left panel), half spindles (middle panel) and microtubule bundles (right panel) are shown. Scale bar, 10μm. (c) Representative images of DNA, Mad2 and Tubulin staining after nocodazole and cold shock (see also Figure 3c). The DNA is shown in the left panel, Mad2 levels in the middle panel and tubulin in the right panel. Scale bar, 5μm (d) CyclinB levels are stabilized by CENP-A chromatin arrays. Chromatin arrays were mixed with CSF extract and released into interphase by addition of calcium. After 80 min, fresh CSF extract supplemented with nocodazole at 10μg/ml was added to drive the extract into mitosis. After 90 min (t 0 min), mitotic exit was induced by adding calcium, and samples were taken 10 min, 20 min, 30 min and 40 min after release. Western blot analysis shows CyclinB levels as an indicator of the cell cycle stage at t 0' (0 min) and t 40' (40 min). Actin levels are shown as a loading control.
(e) Quantification of CyclinB levels over time for the experiment described in (d). Error bars represent SEM, n = 2

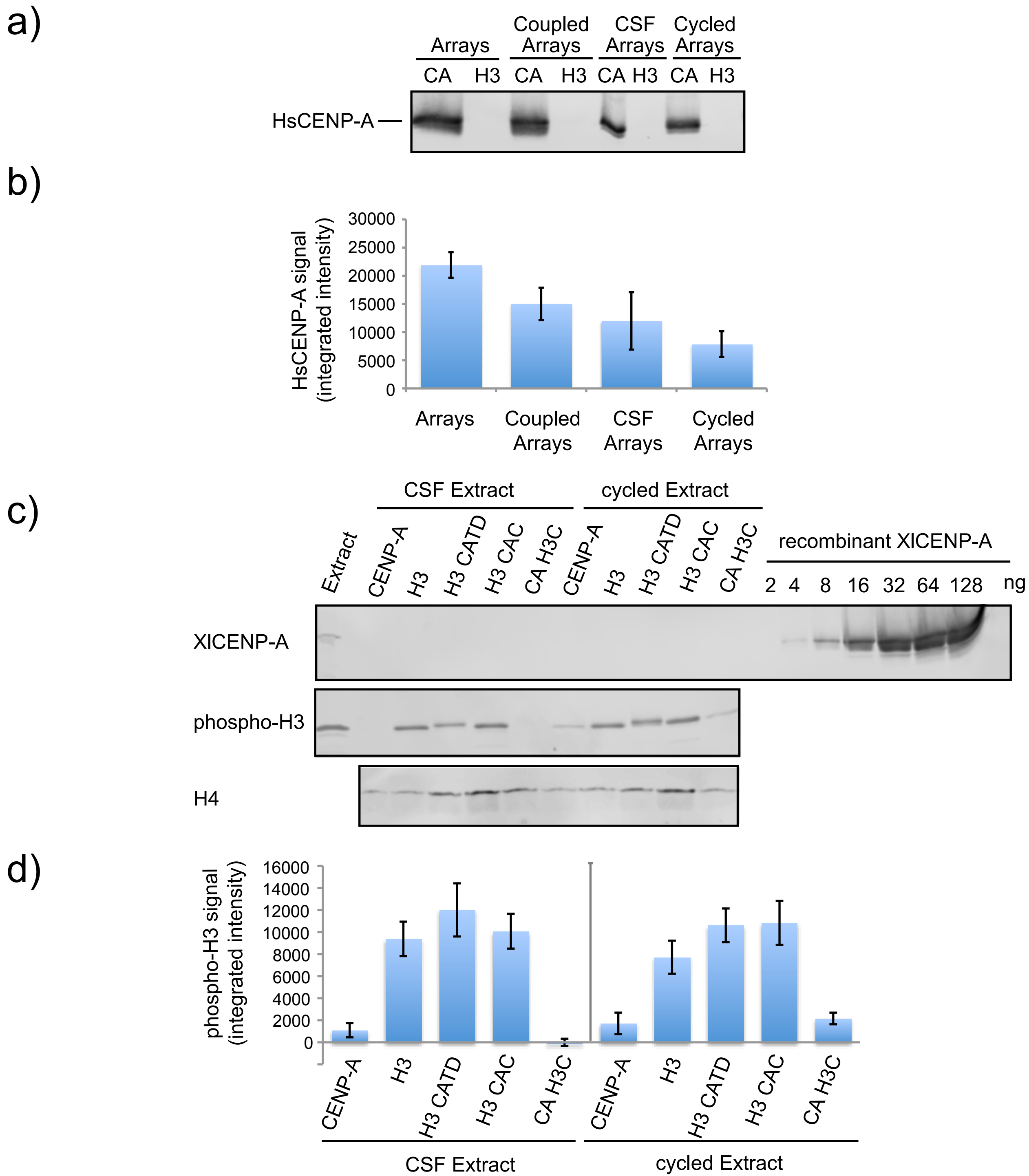
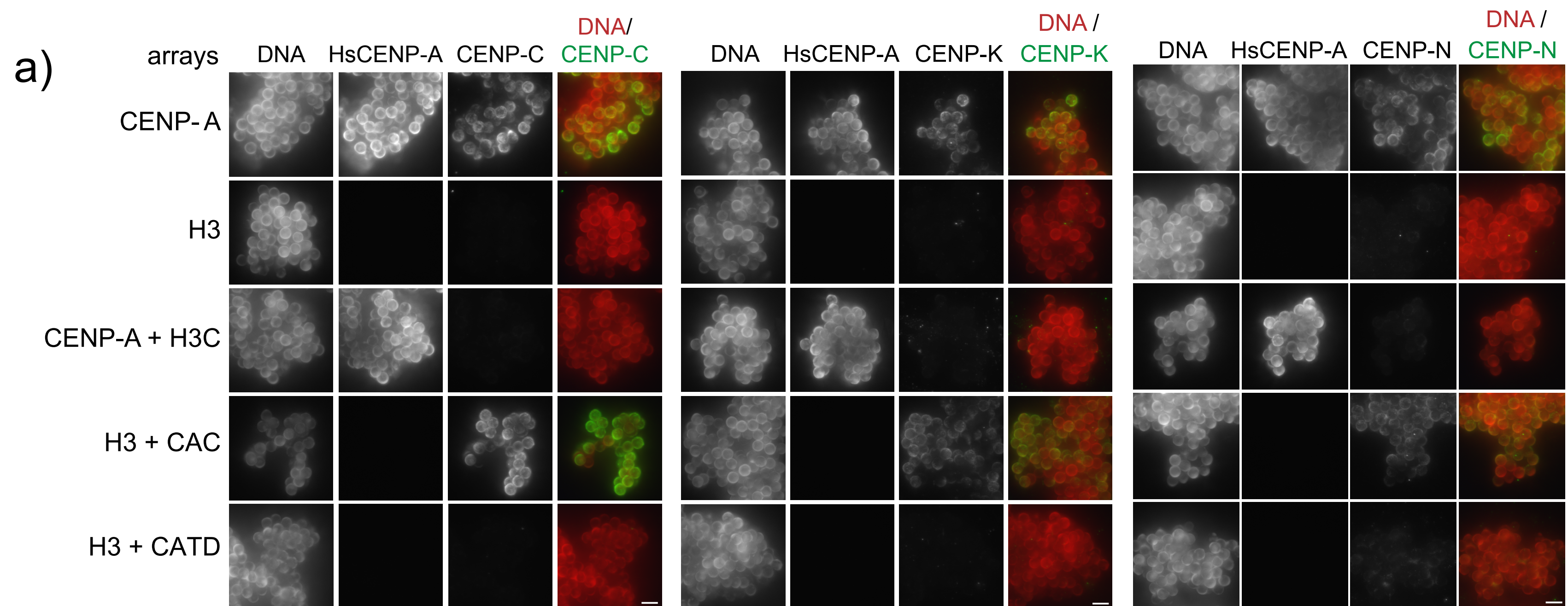


Figure S6

Reconstituted CENP-A chromatin is stably maintained in *Xenopus* egg extracts. (a) HsCENP-A is stably maintained in chromatin arrays. Western blot analysis showing HsCENP-A levels of reconstituted chromatin after assembly (Arrays), after coupling to magnetic beads (Coupled Arrays), after incubation for 60 min in CSF extract (CSF Arrays) and after incubation for 90 min in cycled extracts (Cycled Arrays) using the experimental strategy shown in Figure 2a. (b) Quantification of HsCENP-A levels using western blot analysis. Error bars represent SEM, n = 3. (c) XlCENP-A is not incorporated into chromatin arrays at detectable levels when incubated in *Xenopus* extracts. Western blot analysis of XlCENP-A association with CENP-A, H3 and chimeric chromatin arrays after incubation in CSF extract (CSF Extract) or cycled extract (cycled Extract). The equivalent of ~110ng HsCENP-A was loaded in each lane blotted for α-XlCENP-A. Assuming a loss of < 50%, at least 55ng HsCENP-A is still present in cycled extracts. Thus, 5 -10% exchange of HsCENP-A with XlCENP-A should be detectable when compared to the XlCENP-A dilution series. Phospho-H3 levels are shown for CENP-A, H3 and chimeric chromatin arrays during CSF and cycled egg extract incubation. H4 levels are shown as a loading control. (d) Quantification of phospho-H3 levels determined by western blotting for CENP-A, H3 and chimeric chromatin arrays. Error bars represent SEM, n = 3.



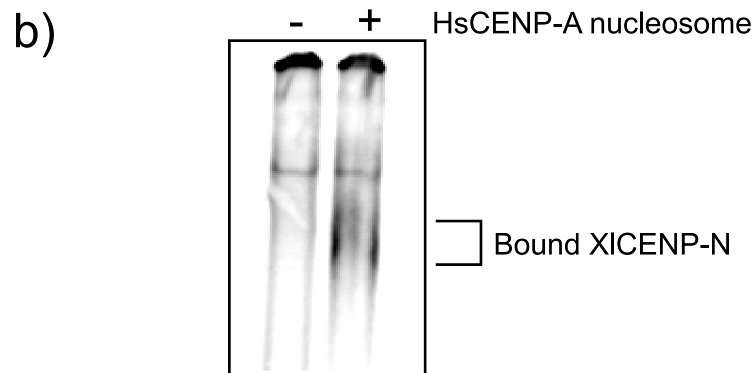


Figure S7

The C-terminus of CENP-A is required for recruitment of CENP-C, CENP-N and CENP-K to chromatin arrays. (a) Representative immunofluorescence images of CENP-C (left panel) CENP-K (middle panel) and CENP-N (right panel) recruitment to CENP-A, H3 and chimeric chromatin arrays. Only CENP-A wild type and CENP-A+H3C arrays show CENP-A staining because the other chimeras lack the epitope recognized by the anti-HsCENP-A antibody. A merge of the DNA (red) and CENP-C, CENP-K and CENP-N, respectively, channels (green) are shown (DNA/CENP-). Scale bar, 5 µm. (b) XlCENP-N binds to HsCENP-A mononucleosomes *in vitro*. 35S-methionine labeled XlCENP-N was incubated in the presence (+) or absence (-) of HsCENP-A mononucleosomes and separated on native acrylamide gels.

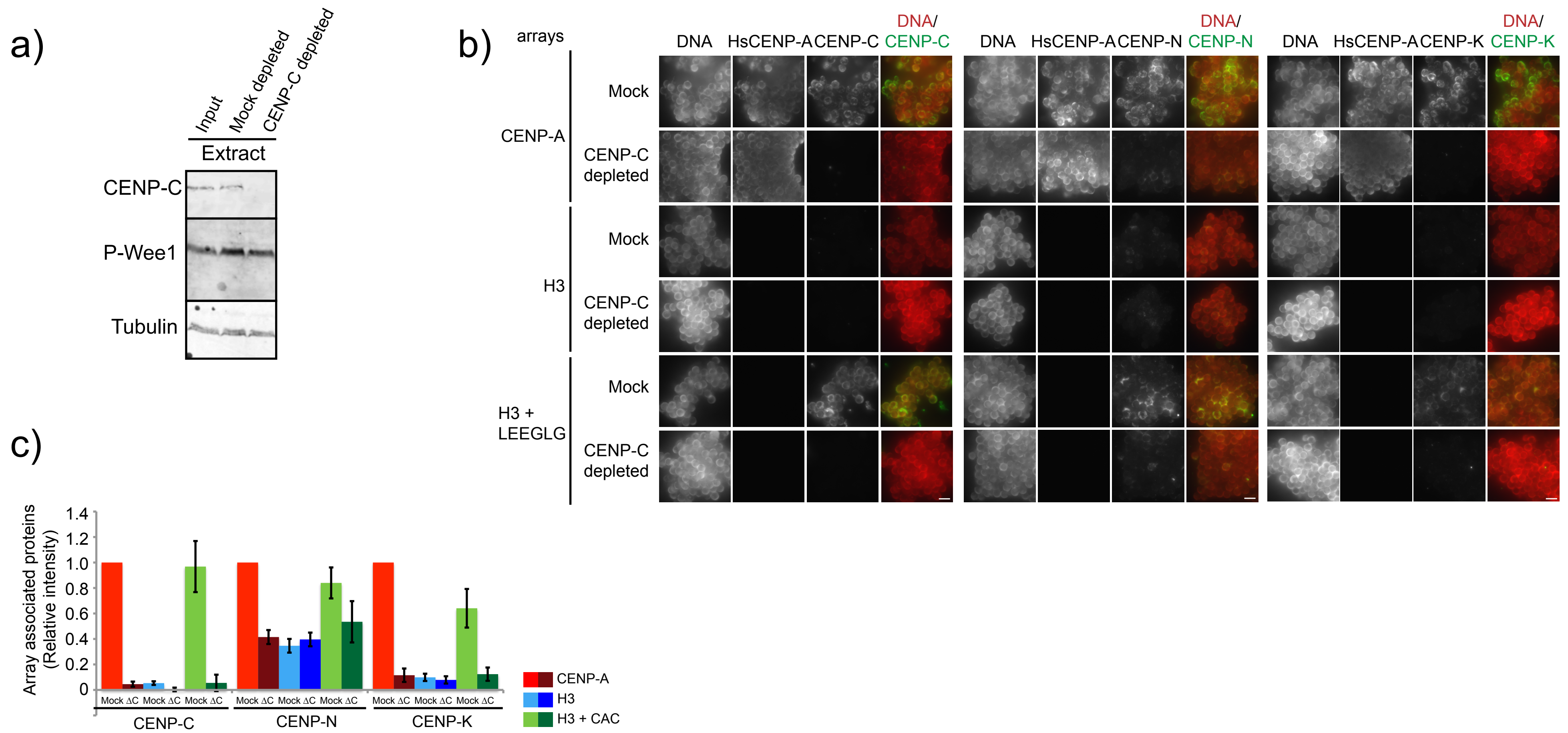


Figure S8

CENP-N and CENP-K are dependent on CENP-C for binding to CENP-A chromatin arrays. (a) A western blot showing efficient depletion of CENP-C from *Xenopus* egg extracts. Equal amounts of extracts were loaded from fresh CSF extract (Input) and after Mock (Mock depleted) and CENP-C depletion (CENP-C depleted). Phospho-Wee1 (P-Wee1) levels show the persistent metaphase arrest during depletion and tubulin levels are shown as a loading control. (b) Representative immunofluorescence images showing CENP-C, CENP-N and CENP-K recruitment to CENP-A wild type (CENP-A), H3 and H3+CAC chromatin arrays in mock (Mock) and CENP-C depleted *Xenopus* egg extracts. The DNA is shown in the left panel. The adjacent panel shows HsCENP-A (the epitope recognized by the anti-HsCENP-A antibody is only present in CENP-A wild type chromatin arrays). The last two panels show CENP-C, CENP-N and CENP-K, respectively and a merge image of the DNA (red) and centromere proteins (green) channels (DNA/CENP-). Scale bar, 5 μm. (c) Quantification of CENP-C, CENP-N and CENP-K recruitment to wild type and chimeric arrays after mock and CENP-C depletion from CSF *Xenopus* extracts. Values are normalized to mock depleted extract containing CENP-A arrays. Error bars represent SEM; n = 3. CENP-C depletion caused a significant loss of centromere protein recruitment to CENP-A and H3+CAC arrays ($p < 0.05$) with the exception of CENP-N binding to H3+CAC arrays ($p=0.21$).

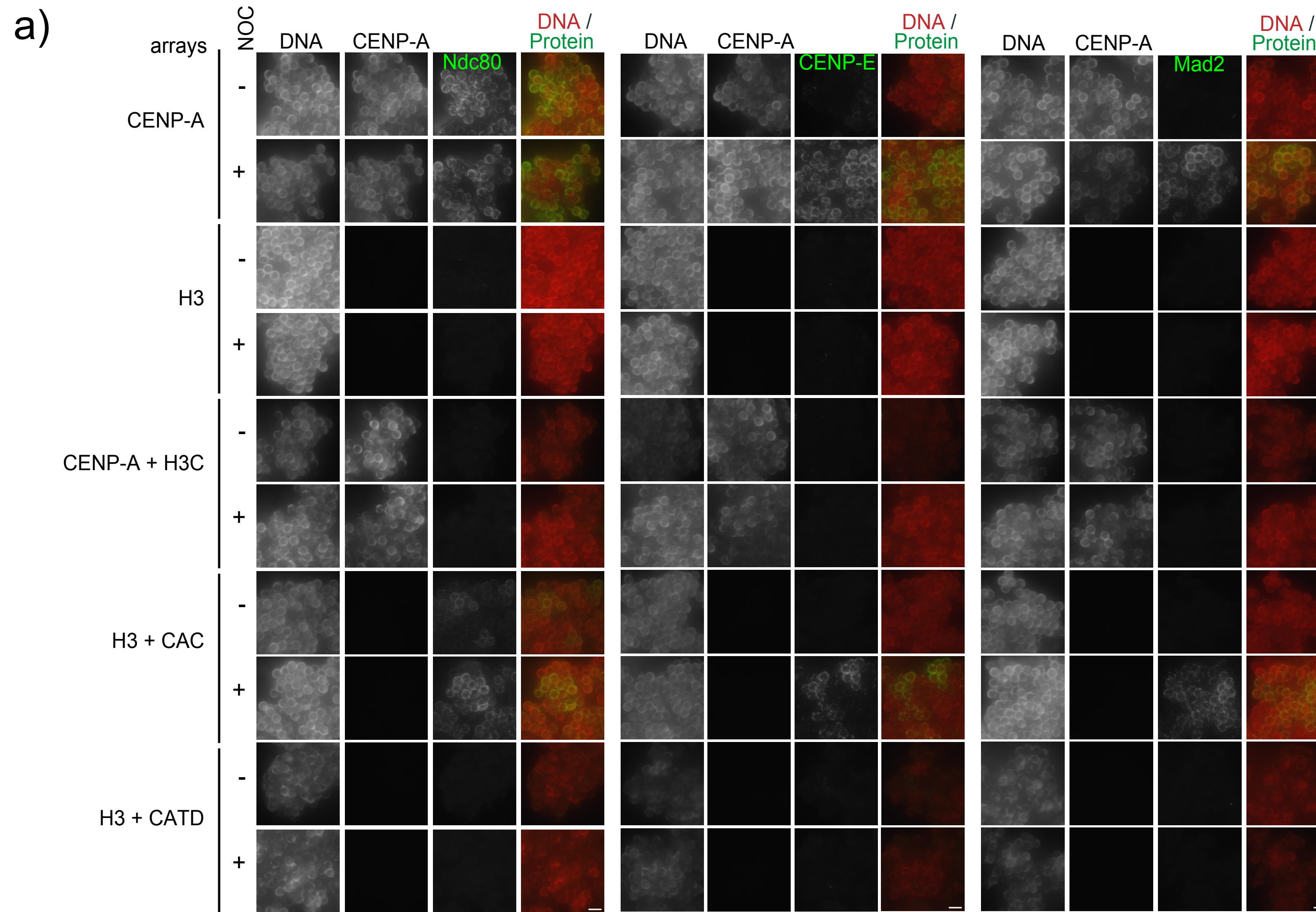
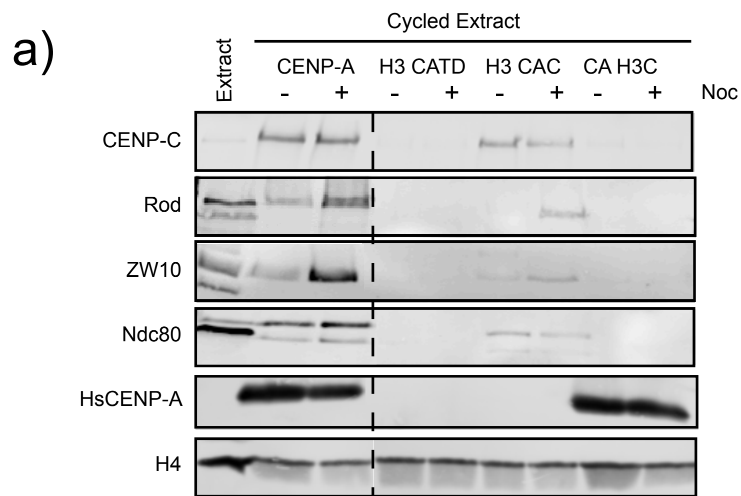


Figure S9

The C-terminal tail of CENP-A is required for kinetochore protein recruitment to chromatin arrays (a) Representative immunofluorescence images of Ndc80, CENP-E and Mad2 recruitment to different chimeric chromatin arrays. The DNA is shown in the left panel. The adjacent panel shows HsCENP-A. Only CENP-A wild type and CENP-A+H3C arrays show CENP-A staining, because the other chimeras lack the epitope recognized by the anti-HsCENP-A antibody. The second panel from the right shows Ndc80, CENP-E and Mad2, respectively. A merge of the DNA (red) and kinetochore proteins (green) channels is shown in the right panel (DNA/Protein). Scale bar, 5 µm.



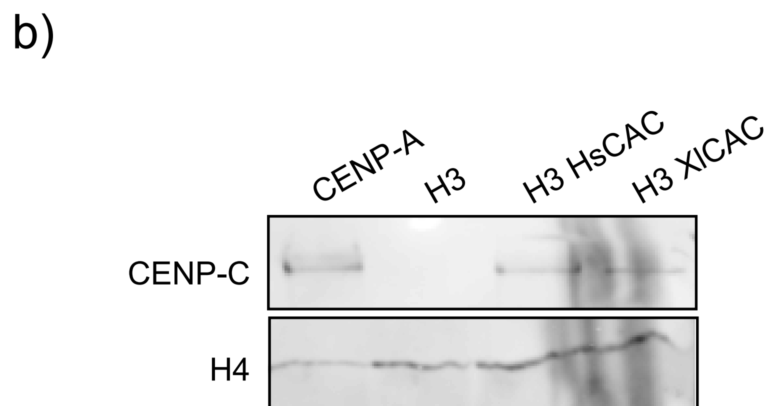


Figure S10
Kinetochore proteins Ndc80, ZW10 and Rod associate with H3+CAC chromatin arrays. (a) Western blot analysis of CENP-C, Rod, ZW10 and Ndc80 recruitment to CENP-A and chimeric chromatin arrays with and without (+/-) nocodazole in cycled egg extracts. H4 and HsCENP-A levels are shown as a loading control. (b) Western blot analysis of CENP-C binding to different types of chromatin arrays in CSF extract. CENP-C binds to CENP-A, H3+HsCAC, H3+XlCAC, but not to H3 chromatin arrays. H4 levels are shown as a loading control.