Westhorpe et al., http://www.jcb.org/cgi/content/full/jcb.201503132/DC1

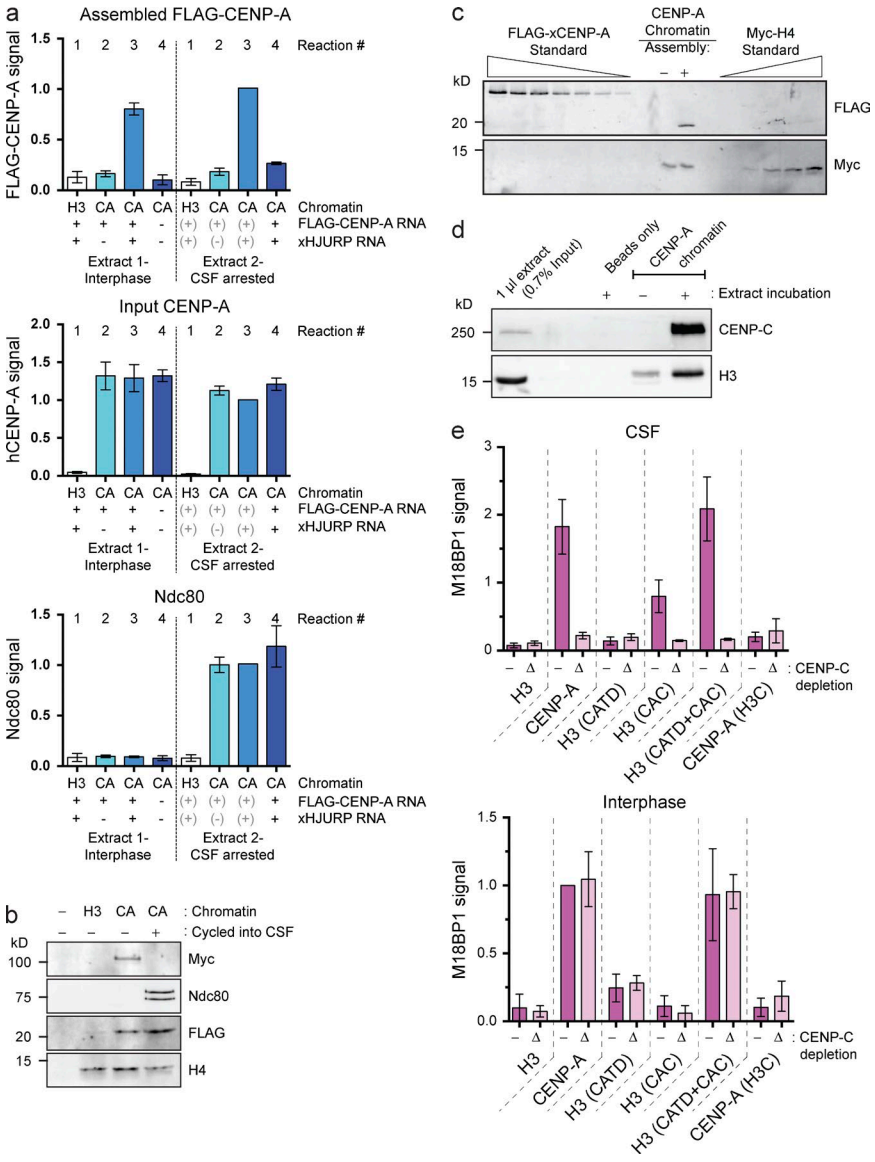

Figure S1. **Characterization of in vitro CENP-A assembly on reconstituted chromatin arrays.** (a) FLAG–xCENP-A signal persists on chromatin arrays cycled into the next mitosis. FLAG–xCENP-A assembly was performed interphase egg extracts as described in Fig. 1 a, and half of the chromatin beads from each reaction were taken to measure FLAG (top), hCENP-A (input nucleosomes, middle), and Ndc80 (M-phase–specific kinetochore marker, bottom). The other half of the reaction was incubated in CSF-arrested *Xenopus* egg extract for 1 h, to move the chromatin back into M phase. Bar colors are consistent between reaction number, also labeled above each bar. Note that FLAG–xCENP-A assembled in interphase in reaction 3 persisted after incubation in CSF extract, whereas chromatin incubated with FLAG–xCENP-A and HJURP RNA only in CSF failed to assemble FLAG–xCENP-A (reaction 4). Input hCENP-A nucleosomes were largely unaffected by FLAG–CENP-A assembly and/or cycling chromatin into M phase, whereas Ndc80 recruitment confirmed that chromatin was cycled into M phase and had assembled kinetochore components. The graphs show the mean values, normalized to signal observed on CENP-A arrays in CSF extract, and error bars show the mean values ± SEM, normalized to reaction 3 in CSF. $n = 3$. (b) Western blot showing that although FLAG–xCENP-A persists in chromatin upon transition of extract into M phase, Myc-HJURP protein is lost. FLAG–xCENP-A assembly was performed in interphase extract by adding Myc-tagged HJURP RNA. Half of the CENP-A arrays were then incubated in CSF egg extract to move them into M phase. Note that although FLAG–xCENP-A signal persisted, consistent with a, Myc-HJURP was lost from CENP-A chromatin in M-phase extract. (c) Example Western blot used to estimate numbers of assembled nucleosomes (FLAG–xCENP-A, top) and input nucleosome number (Myc-H4, bottom) as shown in Fig. 1 d. Known amounts of DNA on which CENP-A/Myc-H4 nucleosomes had been assembled were bound to streptavidin beads (see Fig. 1 A) and incubated in interphase *Xenopus* egg extract supplemented with FLAG–xCENP-A and xHJURP RNA (to promote CENP-A assembly, +) or RNA buffer (no assembly control, –). FLAG–xCENP-A signal was measured against a standard of recombinant, purified FLAG–xCENP-A protein. Note this protein has several additional tags, causing it to migrate slower by SDS-PAGE (see Materials and methods). Recombinant CENP-A/Myc-H4 tetramer was used as a standard protein for measuring input nucleosome number. (d) Histone H3 associated with CENP-A chromatin when chromatin was incubated in extract. Identical amounts of CENP-A chromatin were blotted for histone H3 before or after incubation in *Xenopus* extract (shown by recruitment of CENP-C). Increased H3 signal upon extract incubation, above the cross-reaction to CENP-A histone observed without extract incubation, strongly suggests that H3 nucleosomes assemble on subsaturated chromatin arrays. (e) Chimeric CENP-A/H3 chromatin was added to mock-depleted (–) or CENP-C–depleted extract (Δ) in either CSF-arrested extract (left graph) or extract released into interphase (right graph). M18BP1 levels were assessed by immunofluorescence and normalized to the signal observed on CENP-A arrays in mock-depleted, interphase extract. Note that M18BP1 recruitment to all chimeras in CSF (M phase) is CENP-C dependent. In contrast, during interphase when CENP-A is assembled, M18BP1 recruitment to any CENP-A/H3 chimera is not affected by CENP-C depletion. Note that interphase M18BP1 recruitment to CENP-A arrays is also shown in Fig. 2 a. All bars represent means ± SEM normalized to the signal on CENP-A arrays in interphase; $n = 4$.

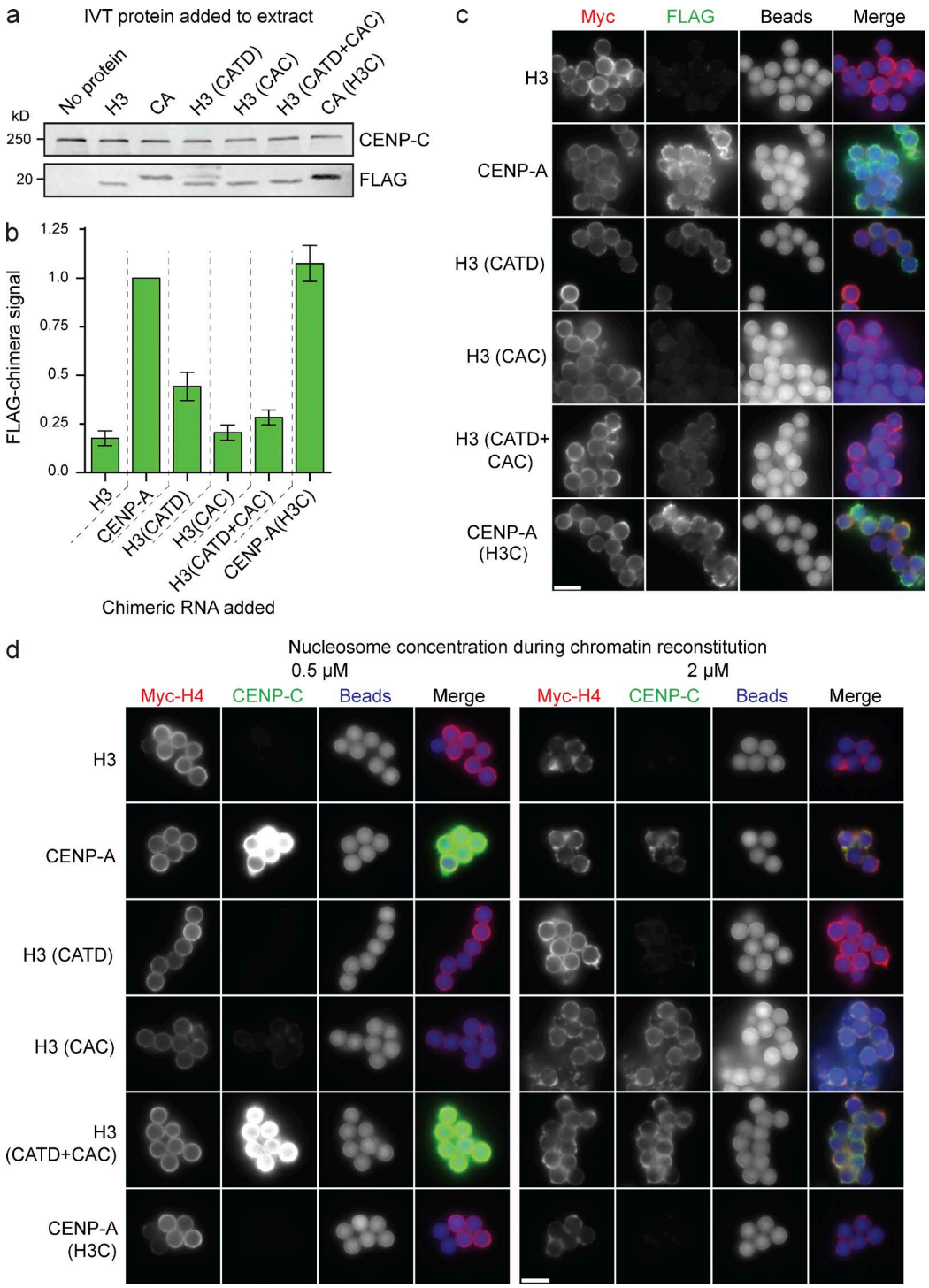

Figure S2. **Assembly of soluble FLAG–CENP-A onto centromeric chromatin depends on the CATD and does not require the CAC.** (a) Protein encoding FLAG epitope–tagged *Xenopus* CENP-A/H3 chimera was produced by IVT and added to released, interphase extract in which xHJURP RNA had already been produced in CSF. Before incubation of extracts with chromatin arrays, a sample of each extract was taken and assessed by Western blot. Note that all six CENP-A/H3 chimeras were produced efficiently by IVT. Although slight differences in IVT protein levels exist, this is not expected to affect FLAG protein assembly, as only a very small proportion of FLAG-histone is assembled from extract (Fig. 1 c). (b) Extract containing IVT produced FLAG epitope xCENP-A/H3 chimera protein was prepared as described in a and each added to low-saturation CENP-A nucleosome arrays. Extract was incubated for 75 min to promote CENP-A assembly, beads were isolated from extract and assessed for assembly of FLAG-chimeric protein. Only wild-type CENP-A and CENP-A(H3C) assembled efficiently onto CENP-A nucleosomes, whereas H3(CATD) partially assembled onto chromatin. Thus, the CAC is not required within soluble CENP-A for assembly of CENP-A into nucleosomes. Note that background levels of assembly were observed on CENP-A nucleosome incubation in extract without addition of xHJURP RNA or on arrays of H3 nucleosomes (not depicted). All bars represent means ± SEM normalized to the signal from assembled FLAG–xCENP-A; $n = 3$. (c) Representative images of the experiment described in b. (d) Full comparison of CENP-C recruitment to CENP-A/H3/Myc-H4 chimeric nucleosome arrays assembled at 0.5 or 2 μM. Representative images (some different to those shown in Fig. 3, c and e) are leveled equivalently, showing that 0.5 μM nucleosome arrays recruit more CENP-C and that CENP-C recruitment only requires the CENP-A C terminus (H3(CAC)) when H3(CAC) is assembled at 2 μM. Bars, 5 μm.

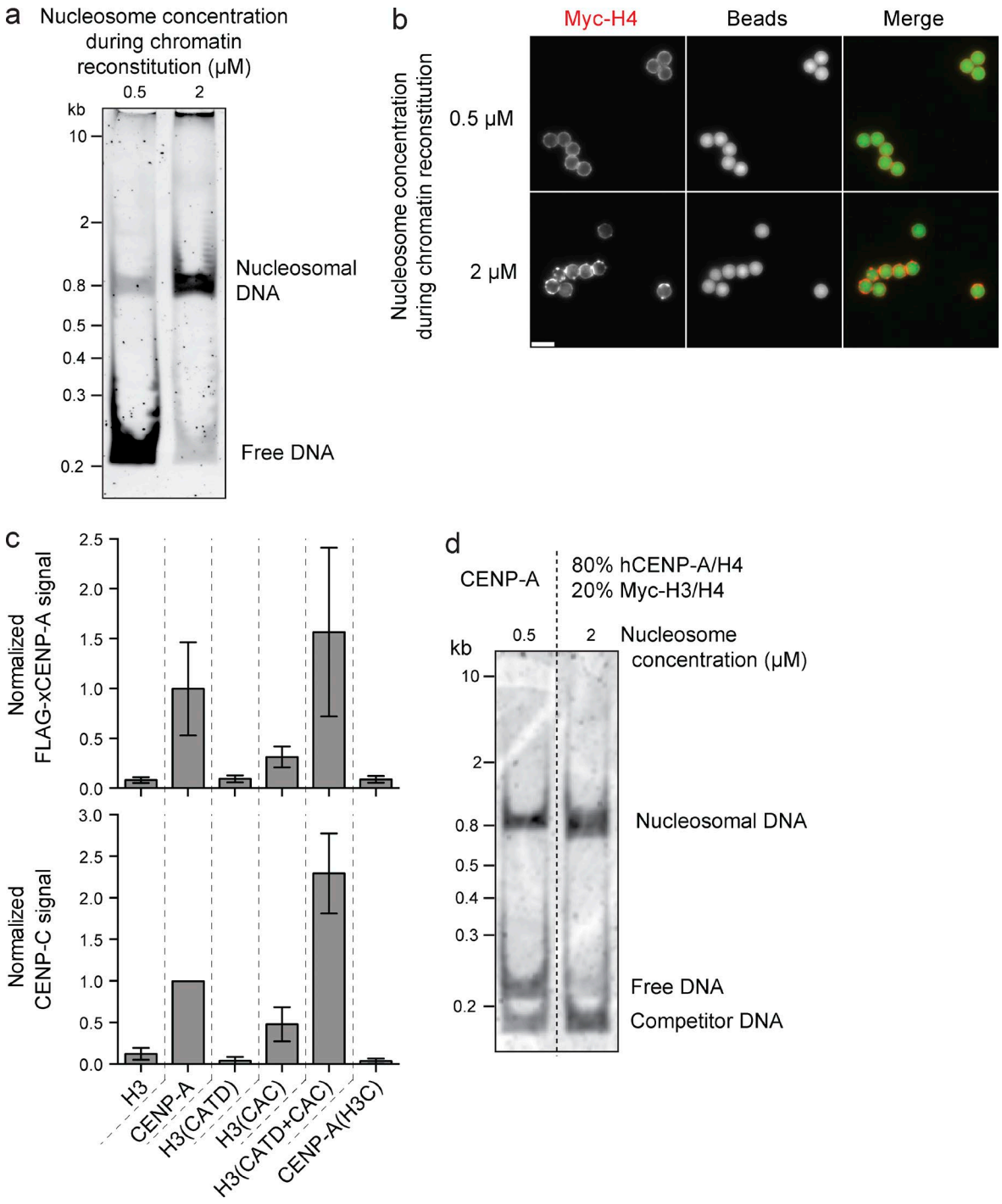

Figure S3. **Characterization of highly saturated, 2 μM nucleosome reconstitutions and FLAG–CENP-A assembly on untagged CENP-A/H4 nucleosome arrays.** (a) SyBr gold–stained native PAGE gel of CENP-A nucleosome arrays reconstituted at a final nucleosome concentration of 0.5 or 2 μM and digested with AvaI, which cuts in between each nucleosome positioning sequence (every 201 bp). Although the same purified histone tetramer/nucleosome positioning site ratio is used in each assembly, 2 μM nucleosome assemblies result in greater saturation of DNA with nucleosomes (nucleosomal DNA band versus free DNA). (b) CENP-A/Myc-H4 nucleosome arrays from a were bound to streptavidin beads and analyzed by immunofluorescence microscopy, without incubating beads in extract. While single plane images of 0.5 μM nucleosome arrays on beads appear as homogenous rings around beads, nucleosome arrays assembled at 2 μM clump on beads. Bar, 5 μm. (c) Quantification of FLAG–xCENP-A assembly and CENP-C localization on CENP-A/H4 chimeric chromatin templates (i.e., not Myc-tagged H4). The graphs show the mean values, normalized to wild-type CENP-A arrays, and error bars represent the means ± SEM between at least four independent experiments. Note that, unlike graphs in Fig. 5, FLAG–xCENP-A and CENP-C signal has not been normalized to input chromatin as no method of detecting input nucleosomes fairly across chimeras is possible with untagged histone H4. (d) SyBr gold–stained native PAGE gel of nucleosome arrays used in experiment described in Fig. 5 e. Arrays of CENP-A nucleosomes or arrays of 80% CENP-A/H4, 20% Myc-H3/H4 were reconstituted at the indicated concentration and digested with AvaI, which cuts in between each nucleosome positioning sequence (every 201 bp). To prevent nonspecific binding of histone to DNA, 161-bp competitor DNA was added to both assemblies. 80% CENP-A/H4 and 20% Myc-H3/H4 arrays are almost completely saturated, as very little free DNA exists. Note that although these assemblies have been analyzed on the same gel and leveled equally, array assemblies in between the lanes shown have been removed.

Supplemental material also includes a ZIP file containing source code for automated image analysis (find_beads) and image processing (panelize)