

# Centromere assembly requires the direct recognition of CENP-A nucleosomes by CENP-N

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**Centromeres are specialized chromosomal domains that direct kinetochore assembly during mitosis. CENP-A (centromere protein A), a histone H3-variant present exclusively in centromeric nucleosomes, is thought to function as an epigenetic mark that specifies centromere identity. Here we identify the essential centromere protein CENP-N as the first protein to selectively bind CENP-A nucleosomes but not H3 nucleosomes. CENP-N bound CENP-A nucleosomes in a DNA sequence-independent manner, but did not bind soluble CENP-A–H4 tetramers. Mutations in CENP-N that reduced its affinity for CENP-A nucleosomes caused defects in CENP-N localization and had dominant effects on the recruitment of CENP-H, CENP-I and CENP-K to centromeres. Depletion of CENP-N using siRNA (short interfering RNA) led to similar centromere assembly defects and resulted in reduced assembly of nascent CENP-A into centromeric chromatin. These data suggest that CENP-N interprets the information encoded within CENP-A nucleosomes and recruits other proteins to centromeric chromatin that are required for centromere function and propagation.**

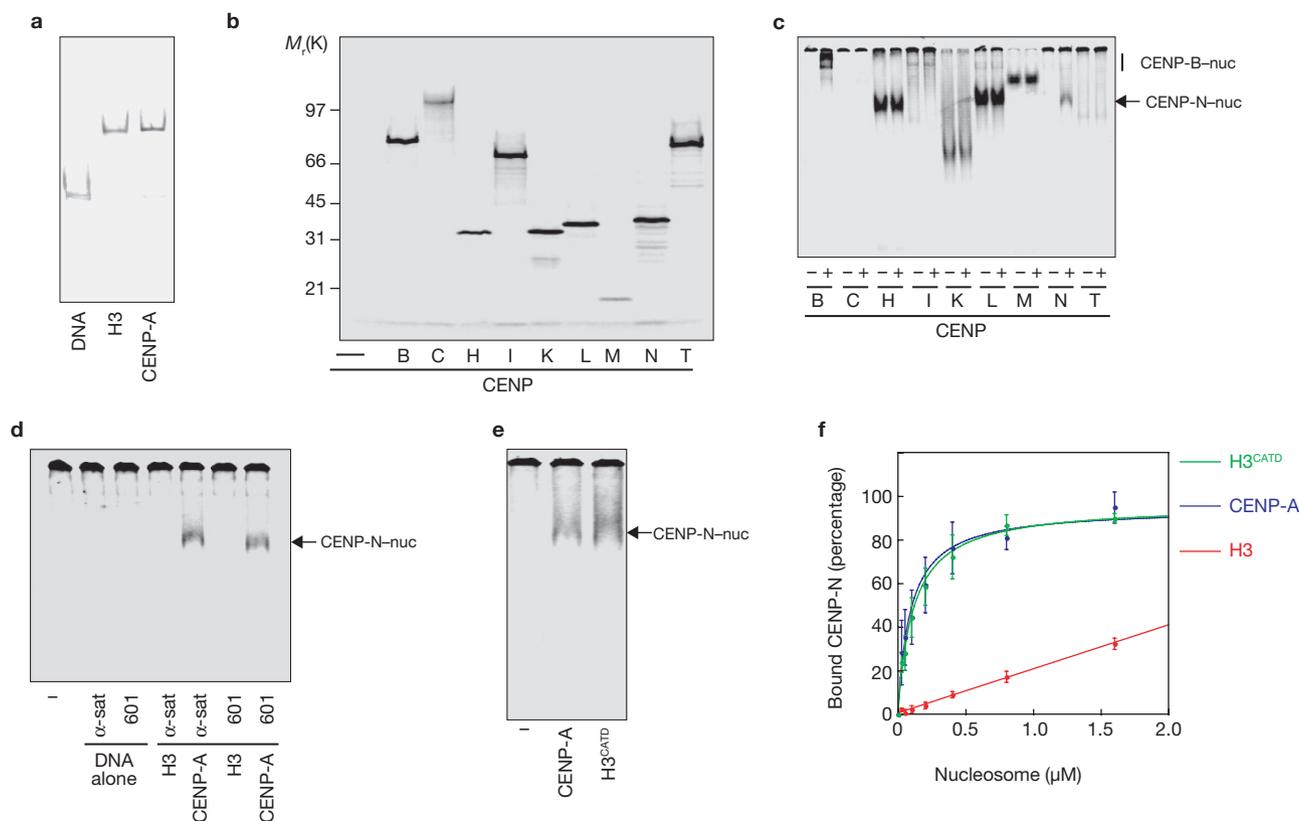
Accurate chromosome segregation during mitosis is essential for the maintenance of genome integrity. Eukaryotic cells have evolved complex machinery to ensure the fidelity of chromosome segregation. Each chromosome directs the assembly of a kinetochore that mediates attachment to the mitotic spindle and is required for microtubule-dependent chromosome movement during mitosis. Kinetochores also function as signalling centres for the mitotic checkpoint, which delays the initiation of anaphase in response to improper chromosome attachment to the mitotic spindle (see ref. 1 for review). The centromere is the region of the chromosome on which the kinetochore is assembled. Human centromeric DNA is composed of repetitive  $\alpha$ -satellite sequences but centromeres are thought to be epigenetically specified, as centromeric DNA is not well conserved between species and no DNA sequences have been identified that are necessary or sufficient for kinetochore function in vertebrates (see ref. 2 for review). In all eukaryotes, centromeric chromatin contains specialized nucleosomes in which histone H3 is replaced by a histone

H3-variant, CENP-A (see ref. 3 for review). This centromere protein is currently the best candidate for the epigenetic mark that specifies centromere identity. It is essential for the centromeric recruitment of most other proteins required for kinetochore function, but the molecular basis for recognition of CENP-A-containing chromatin as the site of kinetochore assembly is poorly understood. Furthermore, the mechanisms that target newly synthesized CENP-A/H4 to established centromeric chromatin to maintain centromere identity have not been determined.

A fundamental limitation in understanding centromere assembly is the lack of well-defined biochemical assays for studying this process. No direct and specific interaction between CENP-A nucleosomes and any of the > 75 proteins that make up a vertebrate mitotic kinetochore has been demonstrated. To identify CENP-A nucleosome-interacting proteins, we developed a simple and rapid binding assay using reconstituted mononucleosomes that contained  $\alpha$ -satellite DNA derived from human centromeres and either histone H3 or CENP-A (Fig. 1a). Potential CENP-A nucleosome-interacting proteins were selected for testing in this assay based on the analysis of kinetochore assembly in several organisms. We focused on the constitutive centromere associated network (CCAN) of proteins, which includes the centromere proteins CENP-C, H, I and K–U, because these proteins are localized to centromeres during interphase and are required for the assembly of functional kinetochores in mitosis. Moreover, their localization, observed by immuno-electron microscopy of CENP-C, to the inner kinetochore plate and the recent demonstration that several of these proteins co-purify with CENP-A mononucleosomes suggests that CCAN proteins are likely to be the chromatin proximal elements of kinetochores<sup>4–7</sup>. We also included CENP-B in our analysis because it has been shown previously to bind directly to a conserved 17-nucleotide motif called the CENP-B box, present in  $\alpha$ -satellite DNA<sup>8</sup>. We expressed CENP-B, C, H, I, and K–U and labelled them with <sup>35</sup>S by coupled *in vitro* transcription and translation (Fig. 1b; Supplementary Information Fig. S1). We then incubated the labelled proteins with or without CENP-A mononucleosomes before resolving the mixtures using native gel electrophoresis. Both CENP-B and CENP-N showed an increased relative migration in the presence of CENP-A nucleosomes assembled with  $\alpha$ -satellite DNA, suggesting that CENP-B and CENP-N

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**Figure 1** CENP-N binds CENP-A nucleosomes. **(a)** Reconstitution of conventional and centromeric nucleosomes. Free 186 base pair human  $\alpha$ -satellite DNA (DNA) or mononucleosomes containing either histone H3 or CENP-A were assembled by salt dialysis and resolved by native gel electrophoresis. **(b)** *In vitro* expression of centromere proteins. Centromere proteins to be used as substrates in nucleosome binding assays were expressed in coupled transcription and translation reactions containing  $^{35}\text{S}$ -methionine. **(c)** CENP-A nucleosome binding assay.  $^{35}\text{S}$ -labelled centromere proteins ( $\sim 1$  nM) were incubated in the presence (+) or absence (-) of reconstituted CENP-A nucleosomes (50 nM) and separated by native gel electrophoresis. Both CENP-B and CENP-N showed CENP-A-nucleosome dependent changes in migration (CENP-B/N-nuc, nucleosome bound CENP proteins). **(d)** CENP-N

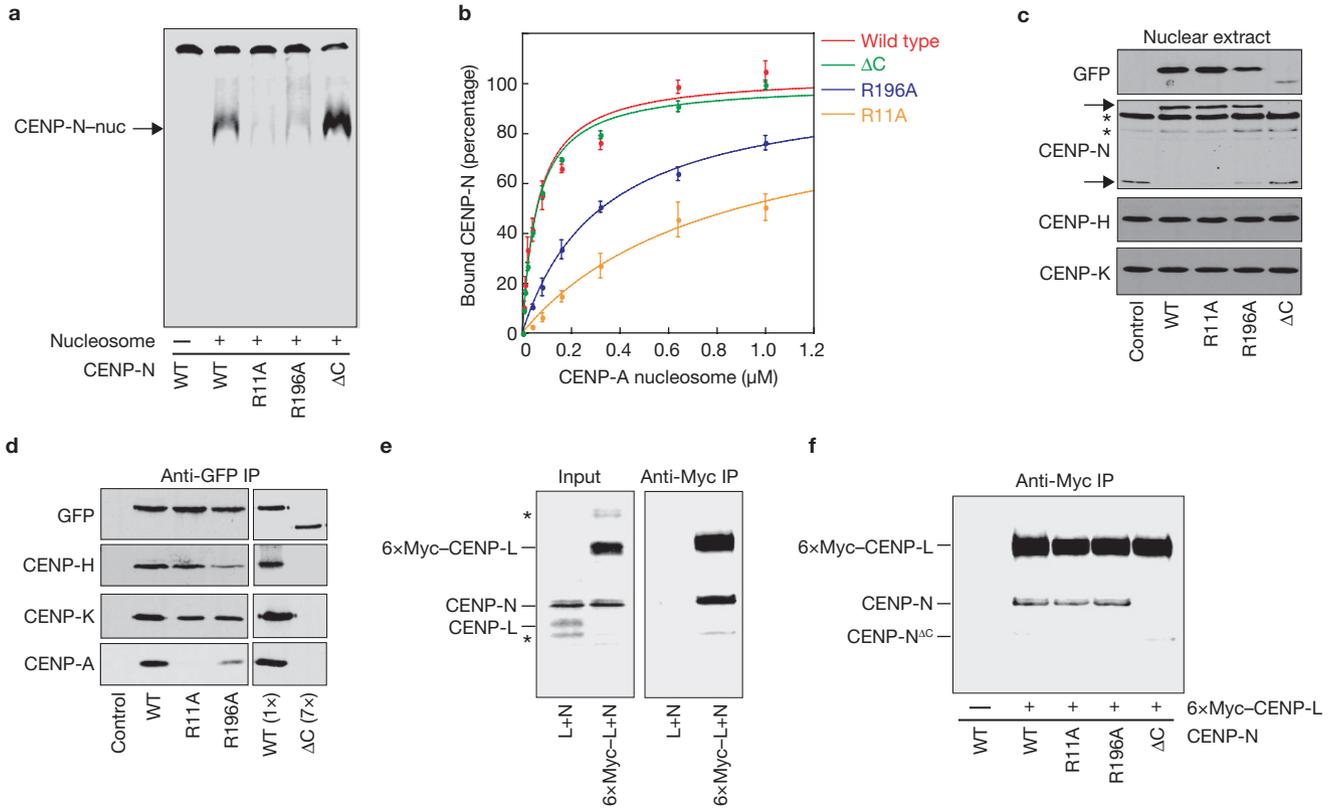
binding to nucleosomes depends on CENP-A and not DNA sequence.  $\alpha$ -satellite DNA ( $\alpha$ -sat) or 601 DNA (601), or H3- or CENP-A-containing nucleosomes reconstituted with  $\alpha$ -satellite or 601 DNA were incubated with  $^{35}\text{S}$ -labelled CENP-N and resolved by native gel electrophoresis. The faster migrating band (arrow) indicates nucleosome-bound CENP-N (- indicates no nucleosome or DNA, as a control). **(e)** Binding of CENP-N to CENP-A nucleosomes occurs through the CATD region of CENP-A. No nucleosome (-) as a control, CENP-A or H3<sup>CATD</sup> nucleosomes reconstituted with  $\alpha$ -satellite DNA were bound to  $^{35}\text{S}$ -CENP-N and assayed as in **c**. **(f)** CENP-N binds with equal affinity to CENP-A and H3<sup>CATD</sup> nucleosomes. CENP-N binding was assayed as in **c** with increasing nucleosome concentration and was quantified according to the  $^{35}\text{S}$ -CENP-N signal in the gel (data are mean  $\pm$  s.e.m.,  $n = 3$ ).

bind directly to CENP-A nucleosomes (Fig. 1c). We did not detect any change in the migration of CENP-C, H, I, K, L, M or O-U (Fig. 1c; Supplementary Information, Fig. S1a). We also tested hMis18 $\alpha$ , hMis18 $\beta$  and M18BP1/hKNL-2, which have been implicated in CENP-A assembly, but we did not detect any interaction with CENP-A nucleosomes in this assay (Supplementary Information, Fig. S1b)<sup>9,10</sup>.

We determined the contribution of DNA sequence and histone protein composition to CENP-B and CENP-N binding of CENP-A nucleosomes by alternately exchanging the nucleosomal DNA and histones. As expected, CENP-B bound to naked  $\alpha$ -satellite DNA as well as both H3 and CENP-A nucleosomes that contained  $\alpha$ -satellite DNA (Supplementary Information, Fig. S1c). CENP-B did not bind the synthetically derived 601 nucleosome positioning sequence or nucleosomes that contained the 601 DNA sequence. In contrast, CENP-N bound equally well to CENP-A nucleosomes that contained either the  $\alpha$ -satellite DNA or the 601 DNA sequence (Fig. 1d). CENP-N did not bind the  $\alpha$ -satellite or 601 DNA fragment or histone H3 nucleosomes assembled on either DNA fragment. CENP-N also bound to CENP-A/H4 tetrasomes reconstituted with  $\alpha$ -satellite DNA that lacked histone H2A and H2B, but did

not specifically bind to soluble CENP-A/H4 tetramers (Supplementary Information, Fig. S1d-f). Thus, CENP-N binds specifically to CENP-A-associated chromatin.

Domain transfer experiments have previously suggested that a contiguous portion of the loop I and helix II region within the histone fold of CENP-A, called the CENP-A targeting domain (CATD), is sufficient for CENP-A function *in vivo*<sup>11,12</sup>. We tested whether the CATD domain was sufficient for CENP-N binding using reconstituted nucleosomes that contained the histone H3<sup>CATD</sup> chimera. CENP-N bound efficiently to H3<sup>CATD</sup>-containing nucleosomes (Fig. 1e), and dose-response experiments revealed that the affinity of CENP-N for H3<sup>CATD</sup> nucleosomes was indistinguishable from the affinity of CENP-N for wild-type CENP-A nucleosomes (apparent  $K_d = 163$  nM  $\pm$  60 versus 169 nM  $\pm$  70, respectively; Fig. 1f; Supplementary Information, Table S1). The CATD domain imparts structural differences to CENP-A nucleosomes, in comparison with H3 nucleosomes, and CENP-A nucleosomes have been suggested to function as an epigenetic mark within chromatin to specify centromere identity<sup>13</sup>. Our data suggest that CENP-N recognizes the unique structural information encoded by the CATD within CENP-A nucleosomes.



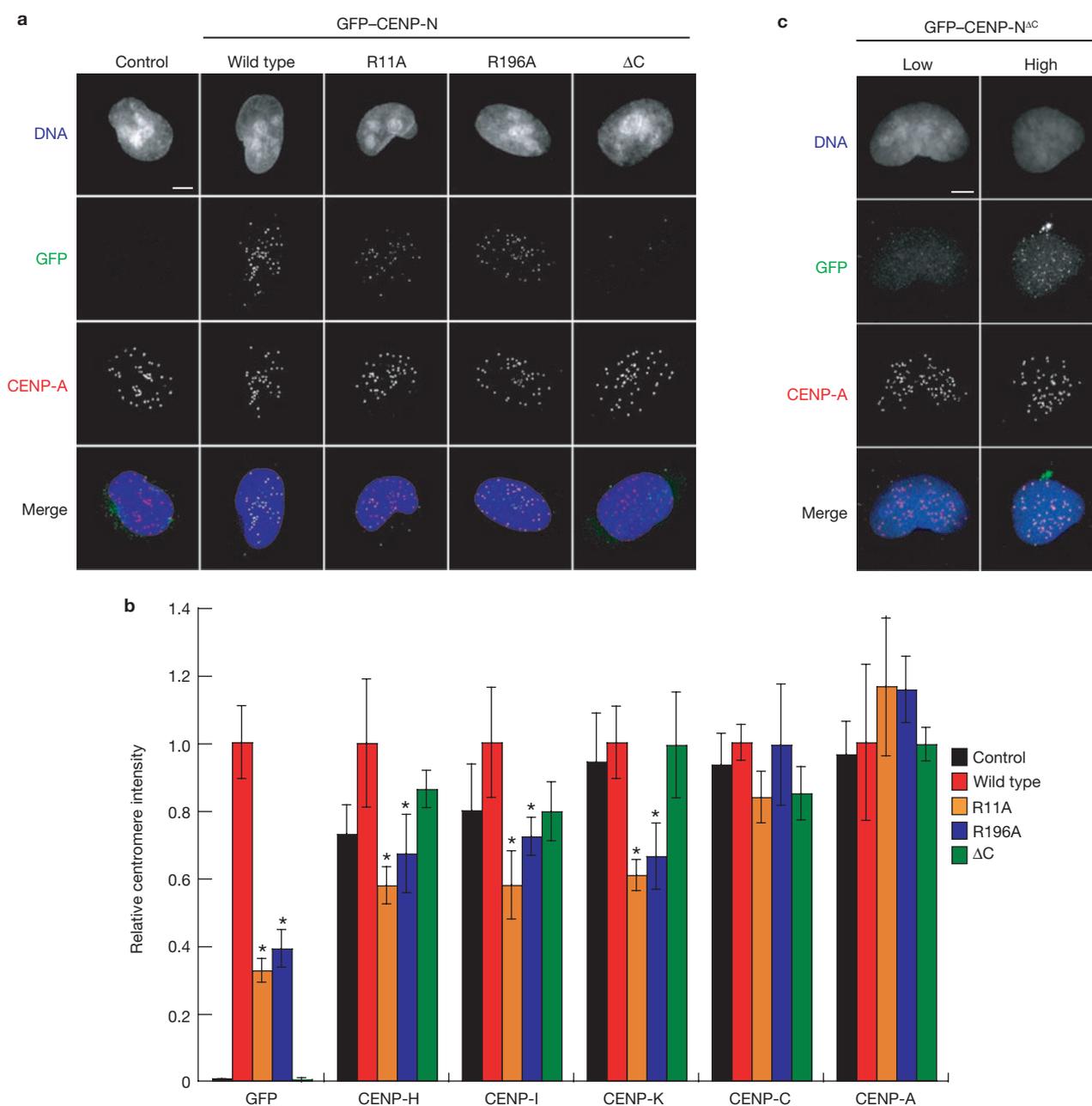
**Figure 2** Identification and characterization of CENP-N mutants defective in CENP-A-nucleosome binding. (a) CENP-N mutants show a range of affinities for CENP-A nucleosomes. CENP-N wild type (WT) or the indicated CENP-N mutants (R11A, R196A and  $\Delta$ C) were expressed and an equal concentration (~1 nM) of each was assayed for the ability to bind CENP-A nucleosomes (CENP-N-nuc, bound CENP). The control reaction (–) lacked CENP-A nucleosomes. (b) Dose-response experiments for each CENP-N mutant were performed as in a, with increasing concentrations of CENP-A nucleosome added to each reaction. Data are mean  $\pm$  s.e.m.,  $n = 3$ . (c) Expression of CENP-N mutants in HEK293 cells. Nuclear extracts from stable HEK293 cell lines expressing GFP-CENP-N (WT) or the indicated GFP-CENP-N mutants were separated by SDS-PAGE and analysed by western blotting using the indicated antibodies. Two nonspecific bands (asterisks) are present in the anti-CENP-N western blot. Upper and lower arrows indicate positions of GFP-CENP-N and endogenous CENP-N, respectively. Quantification

of each band is presented in Supplementary Information, Fig. S4a. (d) Co-immunoprecipitation of CENP-H, K and A from micrococcal-nuclease solubilized chromatin with CENP-N mutants. Anti-GFP immunoprecipitates (IP) from each cell line were probed with the indicated antibodies. Seven times more nuclear extract from the CENP-N $\Delta$ C cell line than the wild-type cell line was required to achieve equal levels of CENP-N in the immunoprecipitations. Quantification of each band is presented in Supplementary Information, Fig. S4b. (e) CENP-N binds to CENP-L. 6xMyc-CENP-L, L and N were expressed and labelled with  $^{35}$ S-methionine and the indicated proteins were mixed at equal stoichiometry. Of each mixture, 20% was resolved as input (left). The remaining 80% was immunoprecipitated with anti-Myc antibodies (right). Two background bands (asterisks) were present in the input reactions. (f) CENP-L binding requires the C terminus of CENP-N. Immunoprecipitations were identical to those in e, except that equal amounts of wild-type CENP-N or the indicated CENP-N mutant was used in each reaction.

Depletion of CENP-N with siRNA causes defects in kinetochore assembly and chromosome congression during metaphase, and results in the loss of most other CCAN components from the centromere<sup>5,14</sup>. However, the depletion of other CCAN subunits, including CENP-H, I and K, results in mitotic phenotypes similar to those caused by CENP-N depletion<sup>4,5,14,15</sup>. Furthermore, CENP-N co-purifies with CENP-H, I, M, K, L and T, which are all likely to be interdependent for centromere localization, as indicated by pairwise dependency relationships<sup>4,5,14,15</sup>. Accordingly, a specific function for CENP-N in recognizing centromeric chromatin *in vivo* cannot be inferred from siRNA-based studies.

To directly determine the role of CENP-A nucleosome binding by CENP-N in centromere assembly, we generated CENP-N mutants that had specifically reduced CENP-A-nucleosome binding affinity. Conserved charged and polar amino acids within CENP-N were changed to alanine, based on sequence alignments of CENP-N orthologues from several species (Supplementary Information, Fig. S2a).

Following an initial characterization of the mutants *in vitro* and *in vivo* (Supplementary Information, Fig. S3 and Table S1), two of the point mutants, CENP-N<sup>R11A</sup> and CENP-N<sup>R196A</sup>, were selected for detailed analysis. Both of these mutants showed reduced CENP-A-nucleosome binding when compared with wild-type CENP-N (Fig. 2a). Dose-response experiments indicated that the CENP-N<sup>R11A</sup> and CENP-N<sup>R196A</sup> mutants had a 6-fold and a 2-fold reduction in CENP-A-nucleosome binding, respectively (Fig. 2b; Supplementary Information, Table S1), suggesting that both Arg 11 and Arg 196 in CENP-N contribute to the recognition of CENP-A nucleosomes. In addition, the carboxyl terminus of CENP-N (amino-acids 289–339 in human CENP-N) is more highly conserved among vertebrates than the rest of the protein (Fig. S2b). We therefore constructed a truncation mutant of CENP-N, lacking the C terminus (CENP-N $\Delta$ C). The CENP-N $\Delta$ C mutant did not affect nucleosome binding when compared with wild-type CENP-N (Fig. 2a, b).

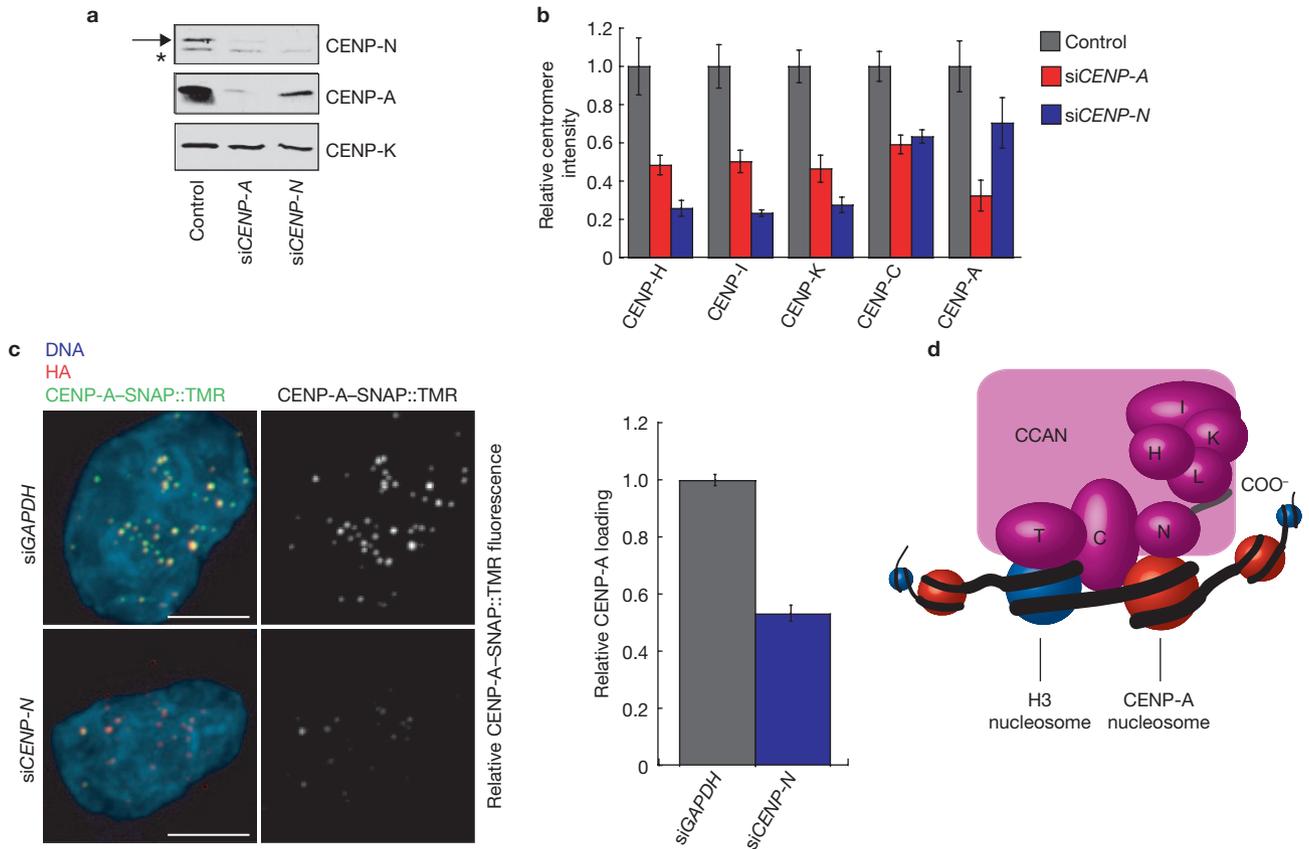


**Figure 3** CENP-N mutants show centromere assembly defects. (a) Representative images of control HEK293 cells and stable cell lines expressing wild-type or the indicated mutant CENP-N. Scale bar, 5  $\mu$ m. (b) CENP-N mutants cause centromere assembly defects. The fluorescence intensity of the indicated centromere protein at individual centromeres in stable cell lines was measured (see Methods). Data are mean  $\pm$  s.e.m.

We generated stable HEK293 cell lines that expressed green-fluorescent protein (GFP) fusions to either wild-type CENP-N or the CENP-N mutants to determine how changing the affinity of CENP-N for the CENP-A nucleosome affected CENP-N localization and centromere assembly *in vivo*. Western blotting indicated that each cell line expressed comparable levels of the respective GFP-CENP-N protein, with the exception of the CENP-N $\Delta C$  mutant, which was reduced  $\sim$ 7-fold compared with wild-type GFP-CENP-N (Fig. 2c; Supplementary Information Fig. S4a). HEK293 cell lines express

from three independent experiments, including > 300 centromeres from > 20 cells for each cell line, \* $P < 0.05$  significance compared with wildtype, as determined by Student's *t*-test. (c) The C terminus of CENP-N is not required for centromere localization. Representative images from transiently transfected HeLa cells show the localization of GFP-CENP-N $\Delta C$  in low and high expressing cells. Scale bar, 5  $\mu$ m.

CENP-N from a single genomic locus under the same promoter (see Methods), suggesting that the decreased protein level is a general property of the CENP-N $\Delta C$  mutant. Interestingly, while the levels of CENP-H and CENP-K were not affected in any of the stable cell lines, the expression of wild-type GFP-CENP-N or either of the two GFP-CENP-N point mutants caused a significant reduction in endogenous CENP-N protein (Fig. 2c). Such a reduction has also been described in cells depleted of CENP-H, suggesting that CENP-N is unstable when not associated with other CCAN components<sup>14</sup>. We



**Figure 4** Depletion of CENP-N affects centromere assembly. (a) CENP-N depletion leads to a reduction in CENP-A levels. HeLa cells were treated with the indicated siRNAs for 56 h and the abundance of the indicated centromere protein was determined by western blotting. Arrow (top) indicates the position of CENP-N. A background band (asterisk) was present in the anti-CENP-N western blot. CENP-K was included as a loading control. (b) CENP-N depletion causes centromere assembly defects. HeLa cells were treated as in a, except that the centromere fluorescence intensity of the indicated protein was measured. Data are mean  $\pm$  s.e.m., from three independent experiments, including > 150 centromeres from > 10 cells for each experiment. (c) Reduced CENP-A assembly after *CENP-N* RNAi transfection. CENP-A-SNAP cells were synchronized, transfected with

siRNAs against *CENP-N* or *GAPDH* and assayed for CENP-A loading by specifically labelling nascent CENP-A-SNAP using TMR-Star as outlined in Supplementary Information, Fig. S5c. Representative images are shown (left). Haemagglutinin (HA) labels a steady state pool of CENP-A-SNAP and is used as a centromere marker. Scale bar, 5  $\mu$ m. Data are mean  $\pm$  s.e.m. from three independent experiments, including the fluorescence intensity of 200 centromeres from 20 cells from each experiment, which was normalized to the *GAPDH* siRNA control. (d) A model depicting the multiple roles of CENP-N in CENP-A-nucleosome recognition and centromere assembly, from the recruitment of CCAN proteins to the CENP-N C-terminal region and centromeric chromatin propagation to the CCAN-dependent regulation of CENP-A-nucleosome assembly.

conclude that wild-type GFP-CENP-N and the GFP-CENP-N<sup>R11A</sup> and GFP-CENP-N<sup>R196A</sup> mutants effectively replaced endogenous CENP-N in these cell lines.

We directly determined the ability of the CENP-N point mutants to interact with other CCAN proteins and with CENP-A nucleosomes *in vivo*. Immunoprecipitation of wild-type GFP-CENP-N showed that GFP-CENP-N bound to CENP-H, K and A nucleosomes (Fig. 2d; Supplementary Information, Fig. S4b). The GFP-CENP-N<sup>R11A</sup> and GFP-CENP-N<sup>R196A</sup> mutants were also associated with CENP-H and CENP-K, although CENP-N<sup>R196A</sup> bound CENP-H less well than wild-type CENP-N. Importantly, the CENP-N<sup>R11A</sup> and CENP-N<sup>R196A</sup> mutants both showed defects in CENP-A-nucleosome binding, the severity of which was consistent with the binding of each mutant to reconstituted CENP-A nucleosomes. Thus, residues Arg 11 and Arg 196 within CENP-N contribute to CENP-A-nucleosome binding *in vitro* and *in vivo*. The comparatively low levels of GFP-CENP-N<sup>ΔC</sup> present in stable cells and the inability of this mutant to downregulate endogenous CENP-N protein levels suggests that the GFP-CENP-N<sup>ΔC</sup>

mutant may not be stably associated with other CCAN proteins. Indeed, GFP-CENP-N<sup>ΔC</sup> did not bind to CENP-H, K or A nucleosomes (Fig. 2d).

To understand the mechanism by which the C terminus of CENP-N mediates CENP-N association with CCAN proteins, we expressed epitope-tagged centromere proteins and untagged CENP-N, in reticulocyte extracts, and performed pairwise binding experiments using anti-Myc immunoprecipitation. Myc-tagged CENP-L, but not untagged CENP-L, efficiently co-precipitated with CENP-N, indicating a direct interaction between CENP-N and CENP-L (Fig. 2e). Myc-tagged CENP-L bound the CENP-N<sup>R11A</sup> and CENP-N<sup>R196A</sup> mutants as efficiently as it bound wild-type CENP-N (Fig. 2f), consistent with the efficient association of these CENP-N mutants with CENP-H and CENP-K *in vivo*. However, the GFP-CENP-N<sup>ΔC</sup> mutant did not bind to CENP-L. These data suggest that CENP-N associates with other CCAN components through a direct interaction with CENP-L that requires the highly conserved C terminus of CENP-N.

We compared the localization of CENP-N<sup>R11A</sup>, CENP-N<sup>R196A</sup> and CENP-N<sup>ΔC</sup> with wild-type CENP-N. Wild-type GFP-CENP-N localized

exclusively to centromeres as indicated by colocalization with endogenous CENP-A (Fig. 3a). CENP-N<sup>R11A</sup> and CENP-N<sup>R196A</sup> also localized to centromeres but did so inefficiently. Quantification showed that the levels of the CENP-N<sup>R11A</sup> and CENP-N<sup>R196A</sup> at centromeres was reduced to  $32 \pm 4\%$  and  $39 \pm 6\%$ , respectively, from the wild type (Fig. 3b). The difference in localization efficiency between the CENP-N<sup>R11A</sup> and CENP-N<sup>R196A</sup> mutants was not as great as would be predicted from their relative CENP-A-nucleosome binding affinities (Fig. 2b, d), suggesting that the other CCAN proteins probably contribute to CENP-N localization efficiency *in vivo*. Nevertheless, these data show that mutations that reduce CENP-A-nucleosome binding by CENP-N result in quantitative defects in the centromere-specific localization of CENP-N.

We did not detect the CENP-N<sup>ΔC</sup> mutant at centromeres in our stable cell line, suggesting that association with other CCAN subunits is an important step in the recruitment of CENP-N to centromeres (Fig. 3a, b). However, transiently transfected cells expressing the GFP-CENP-N<sup>ΔC</sup> mutant from a strong promoter occasionally contained detectable levels of the mutant protein at centromeres (Fig. 3c). Thus, the C terminus of CENP-N is not absolutely required for CENP-N centromere localization. Instead, our data indicate that association with other CCAN components stabilizes CENP-N and probably increases the efficiency of CENP-N centromere localization.

Mutations in CENP-N that affected CENP-A-nucleosome binding caused dominant defects in centromere assembly in our stable cell lines. Quantification of CENP-H, I and K in cells expressing CENP-N<sup>R11A</sup> and CENP-N<sup>R196A</sup> indicated that the levels of each protein at centromeres in interphase cells was significantly reduced when compared with levels in cells expressing wild-type CENP-N (Fig. 3b). Thus, reducing the level of CENP-N at centromeres led to a reduction in the levels of a subset of other CCAN subunits. The defect in CENP-H, I and K localization in the mutant cells was not as severe as that of CENP-N itself, suggesting that a small amount of remaining endogenous CENP-N may also contribute to centromere assembly in these cell lines (Fig. 3b). CENP-N<sup>R11A</sup> and CENP-N<sup>R196A</sup> did not alter the levels of CENP-A or CENP-C at centromeres.

Next, we examined the dependence of CENP-H, I and K localization on CENP-N by depleting CENP-N with siRNA in HeLa cells (Fig. 4a; Supplementary Information, Fig. S5a). CENP-N depletion led to a substantial reduction in the levels of CENP-H, I and K at centromeres (Fig. 4b), consistent with previous studies demonstrating CENP-H reduction in cells depleted of CENP-N (ref. 14). The localization defects of CENP-H, I and K in CENP-N-depleted cells were more severe than in cells depleted of CENP-A, indicating that these defects are not an indirect consequence of the reduced CENP-A levels in the CENP-N-depleted cells (Fig. 4b, see below). CENP-C localization to centromeres was also reduced in CENP-N-depleted cells, but not to the same extent as other CCAN proteins (Fig. 4b). Thus, the dependence of CCAN protein localization on CENP-N function was similar between CENP-N-depleted cells and stable cell lines expressing CENP-N mutants with CENP-A-nucleosome binding defects.

Depletion of CENP-N caused a reduction in total levels of CENP-A (Fig. 4a) and CENP-A levels at centromeres (Fig. 4b; Supplementary Information, Fig. S5b). CENP-N is therefore required for the maintenance of centromeric chromatin. Similar defects have been described in *Schizosaccharomyces pombe* cells with mutations in the CENP-N orthologue Mis15, suggesting that CENP-N function is evolutionarily

conserved<sup>16</sup>. CENP-H, I and K are required for the deposition of newly synthesized CENP-A at centromeres<sup>4</sup>. We therefore asked whether the reduction in CENP-A levels within centromeric chromatin in CENP-N-depleted cells was due to a failure to load new CENP-A at centromeres. To address this we used the pulse labelling method, based on the SNAP-tag, to determine the fate of newly synthesized protein. Using this strategy, the assembly of nascent CENP-A has been shown previously to be restricted to early G1 phase<sup>17</sup>. The siRNA-mediated reduction of CENP-N levels resulted in a significant reduction in the recruitment of newly synthesized CENP-A-SNAP to centromeres (Fig. 4c; Supplementary Information, Fig. S5c), indicating that the loss of steady-state levels of centromeric CENP-A is caused, at least in part, by a defect in CENP-A assembly.

We have identified CENP-N as the first protein to bind specifically to CENP-A nucleosomes and shown that the direct binding of CENP-A nucleosomes by CENP-N is required for centromere assembly. The DNA sequence-independent binding of CENP-A nucleosomes by CENP-N suggests that CENP-N recognizes the epigenetic mark in chromatin that specifies centromere identity. Importantly, whereas CENP-N targets directly to CENP-A nucleosomes, we found that CENP-N itself is required for recruiting new CENP-A to the centromere, suggesting that CENP-N is part of a feedback loop responsible for propagating centromeric chromatin in dividing cells (Fig. 4d).

CENP-N mutants with a 2-fold or 6-fold reduction in apparent binding affinity for CENP-A nucleosomes, compared with wild-type CENP-N, localized equally efficiently to centromeres *in vivo*, suggesting that CCAN proteins other than CENP-N may also provide direct interactions with chromatin that are important for centromere assembly. The interdependence of several CCAN proteins, including CENP-N and CENP-T, for centromere localization is consistent with this possibility<sup>5</sup>. A complex of CENP-T and CENP-W was recently shown to bind directly to DNA *in vitro* and it associates with histone H3-containing nucleosomes<sup>18</sup>. CENP-N and the CENP-T-W complex are therefore likely to cooperate in providing multiple distinct chromatin contacts that are required for the localization of a subset of other CCAN proteins, including CENP-H, I and K, to centromeres.

CENP-C assembly at centromeres is less sensitive to CENP-N depletion than the other CCAN proteins we examined, consistent with previous results suggesting that CENP-I and CENP-C are independently recruited to centromeric chromatin in human cells<sup>19</sup>. Nevertheless, CENP-A is required for both CENP-N and CENP-C centromere localization, indicating that several centromere localization pathways downstream of CENP-A exist<sup>5,20</sup>. CENP-C has been shown previously to bind directly to DNA *in vitro*, but it is unclear how such an activity could translate into the centromere-specific localization observed for CENP-C *in vivo*<sup>21,22</sup>. Identifying the molecular mechanisms by which CENP-C is recruited to CENP-A chromatin in the absence of CENP-N, and understanding how these distinct centromere-recognition pathways are integrated at the level of chromatin will provide important insights into centromere assembly and structure. □

## METHODS

Methods and any associated references are available in the online version of the paper at <http://www.nature.com/naturecellbiology/>.

Note: Supplementary Information is available on the Nature Cell Biology website.

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#### AUTHOR CONTRIBUTIONS

C.W.C. and A.F.S. designed the experiments and wrote the manuscript; C.W.C. performed all the experiments except those presented in Figure 4c, which were performed by M.C.C.S. and L.E.T.J.; and K.G. purified histones and helped with nucleosome assembly.

#### COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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- Cheeseman, I. M. & Desai, A. Molecular architecture of the kinetochore-microtubule interface. *Nature Rev. Mol. Cell Biol.* **9**, 33–46 (2008).
- Choo, K. H. Domain Organization at the Centromere and Neocentromere. *Dev. Cell* **1**, 165–177 (2001).
- Carroll, C. W. & Straight, A. F. Centromere formation: from epigenetics to self-assembly. *Trends Cell Biol.* **16**, 70–78 (2006).
- Okada, M. *et al.* The CENP-H-I complex is required for the efficient incorporation of newly synthesized CENP-A into centromeres. *Nature Cell Biol.* **8**, 446–457 (2006).
- Foltz, D. R. *et al.* The human CENP-A centromeric nucleosome-associated complex. *Nature Cell Biol.* **8**, 458–469 (2006).
- Izuta, H. *et al.* Comprehensive analysis of the ICEN (Interphase Centromere Complex) components enriched in the CENP-A chromatin of human cells. *Genes Cells* **11**, 673–684 (2006).
- Saitoh, H. *et al.* CENP-C, an autoantigen in scleroderma, is a component of the human inner kinetochore plate. *Cell* **70**, 115–125 (1992).
- Masumoto, H., Masukata, H., Muro, Y., Nozaki, N. & Okazaki, T. A human centromere antigen (CENP-B) interacts with a short specific sequence in aliphoid DNA, a human centromeric satellite. *J. Cell Biol.* **109**, 1963–1973 (1989).
- Fujita, Y. *et al.* Priming of centromere for CENP-A recruitment by human hMis18 $\alpha$ , hMis18 $\beta$ , and M18BP1. *Dev. Cell* **12**, 17–30 (2007).
- Maddox, P. S., Hyndman, F., Monen, J., Oegema, K. & Desai, A. Functional genomics identifies a Myb domain-containing protein family required for assembly of CENP-A chromatin. *J. Cell Biol.* **176**, 757–763 (2007).
- Black, B. E. *et al.* Structural determinants for generating centromeric chromatin. *Nature* **430**, 578–582 (2004).
- Black, B. E. *et al.* Centromere identity maintained by nucleosomes assembled with histone H3 containing the CENP-A targeting domain. *Mol. Cell* **25**, 309–322 (2007).
- Black, B. E., Brock, M. A., Bedard, S., Woods, V. L. Jr & Cleveland, D. W. An epigenetic mark generated by the incorporation of CENP-A into centromeric nucleosomes. *Proc. Natl Acad. Sci. USA* **104**, 5008–5013 (2007).
- McClelland, S. E. *et al.* The CENP-A NAC/CAD kinetochore complex controls chromosome congression and spindle bipolarity. *EMBO J.* **26**, 5033–5047 (2007).
- Cheeseman, I. M., Hori, T., Fukagawa, T. & Desai, A. KNL1 and the CENP-H/I/K complex coordinately direct kinetochore assembly in vertebrates. *Mol. Biol. Cell* (2007).
- Hayashi, T. *et al.* Mis16 and Mis18 are required for CENP-A loading and histone deacetylation at centromeres. *Cell* **118**, 715–729 (2004).
- Jansen, L. E., Black, B. E., Foltz, D. R. & Cleveland, D. W. Propagation of centromeric chromatin requires exit from mitosis. *J. Cell Biol.* **176**, 795–805 (2007).
- Hori, T. *et al.* CCAN Makes Multiple contacts with centromeric DNA to provide distinct pathways to the outer kinetochore. *Cell* **135**, 1039–1052 (2008).
- Liu, S. T., Rattner, J. B., Jablonski, S. A. & Yen, T. J. Mapping the assembly pathways that specify formation of the trilaminar kinetochore plates in human cells. *J. Cell Biol.* **175**, 41–53 (2006).
- Goshima, G., Kiyomitsu, T., Yoda, K. & Yanagida, M. Human centromere chromatin protein hMis12, essential for equal segregation, is independent of CENP-A loading pathway. *J. Cell Biol.* **160**, 25–39 (2003).
- Yang, C. H., Tomkiel, J., Saitoh, H., Johnson, D. H. & Earnshaw, W. C. Identification of overlapping DNA-binding and centromere-targeting domains in the human kinetochore protein CENP-C. *Mol. Cell Biol.* **16**, 3576–3586 (1996).
- Trauzzi, S. *et al.* *In vivo* functional dissection of human inner kinetochore protein CENP-C. *J. Structural Biology* **140**, 39–48 (2002).

## METHODS

**cDNA isolation and expression.** cDNAs encoding CENP-C, N, K, T, and I were amplified from HeLa cell mRNA with gene-specific oligonucleotides using the Super-Script III kit (Invitrogen) and cloned by blunt-end ligation into pCR4 Blunt-TOPO using the Zero-Blunt cloning kit (Invitrogen) according to the manufacturers instructions. cDNAs encoding CENP-B, CENP-H, CENP-L, CENP-M, CENP-O, CENP-P, CENP-Q, CENP-R, CENP-S, Mis18 $\alpha$ , Mis18 $\beta$  and Mis18BP1/KNL2 were produced by gene synthesis (DNA 2.0, Palo Alto). All cDNAs were sequenced and subcloned into the AscI and PacI sites of a modified pCS2+ based plasmid for coupled transcription and translation in reticulocyte lysates (Promega). <sup>35</sup>S-methionine (Amersham) was added to specifically label each protein. The abundance of the centromere proteins was estimated using western blotting.

**Histone expression.** Histones H2A, H2B, H3 and H4 were expressed, purified and refolded as described previously<sup>23</sup>, except that the soluble H3/H4 tetramer and H2A/H2B dimer, rather than the histone octamer, were assembled and frozen on liquid nitrogen. CENP-A was expressed as a soluble tetramer with histone H4 as described previously<sup>11</sup>. Briefly, BL21 bacterial cells (6 l), transformed with a bi-cistronic vector encoding human CENP-A and *Xenopus laevis* histone H4, were grown to optical density (OD) 600 = 0.2 at 37 °C. The culture was then switched to 23 °C and grown until reaching OD600 = 0.6, at which point IPTG (isopropyl- $\beta$ -D-thiogalactoside) was added to 0.3 mM for 6 h to induce protein expression. The cells were spun down and frozen directly on liquid nitrogen. Cell pellet was resuspended in 100 ml of lysis buffer (10 mM KPO<sub>4</sub>, 0.9 M NaCl and 10 mM  $\beta$ -mercaptoethanol — BME — and 1 mM phenylmethylsulphonyl fluoride — PMSF — at pH 6.8), sonicated and centrifuged at 100,000g to clarify the extract. The high-speed supernatant was added to a hydroxyapatite column, which was washed with five column volumes of lysis buffer and eluted with a 0.9 M to 3.5 M NaCl gradient in KPO<sub>4</sub> (10 mM at pH 6.8). Soluble CENP-A/H4 typically elutes in a broad peak between 1.5 and 3.5 M NaCl. Fractions containing CENP-A/H4 tetramers were pooled and dialysed two times against SP-loading buffer (10 mM Tris-HCl, 0.75 M NaCl, 10 mM BME and 0.5 mM EDTA at pH 7.4). The dialysate was loaded onto a 1 ml Hi-Trap SP column (Amersham) and eluted with a 0.75 to 2 M NaCl gradient in Tris-HCl (10 mM) and EDTA (0.5 mM at pH 7.4). Fractions containing CENP-A/H4 were pooled, concentrated and added to a Sephadex 200 gel-filtration column equilibrated with Tris-HCl (10 mM), NaCl (2 M) and EDTA (0.5 mM at pH 7.4). Peak fractions were pooled, concentrated and frozen down for nucleosome reconstitution. Approximately 5 mg of CENP-A/H4 tetramer can be expected from 6 l of cells.

**Nucleosome reconstitution.** DNA (186-bp) containing the centromere repeat or the 601 nucleosome-positioning sequence was generated by polymerase chain reaction (PCR). CENP-A/H4 tetramer, H2A/H2B dimer and DNA were mixed at a stoichiometry of 1.1:2.2:1 in high-salt buffer (10 mM Tris-HCl, 2 M NaCl and 0.5 mM EDTA at pH 7.4) and the salt concentration was slowly lowered to 2.5 mM NaCl over ~60 h by gradient dialysis. Mononucleosomes were then purified on a 5 ml 5–30% glycerol gradient as described previously<sup>23</sup>. Fractions containing pure mononucleosomes were concentrated and stored at 4 °C.

**Gel shift assays.** Centromere proteins were run out alone or in the presence of the indicated nucleosome on 5% acrylamide gels in 0.5 $\times$  TBE for 70 min at 10 mA. Typical binding reactions were carried out for 30 min at room temperature and contained the indicated concentrations of nucleosome and 0.5–1  $\mu$ l of *in vitro* transcription/translation (ivt) mix in Tris-HCl (10 mM) and glycerol (20%) at pH 7.4. Binding reactions were loaded directly on the gel. After electrophoresis, gels were first stained with ethidium bromide or SYBR-gold (Invitrogen) to visualize and/or quantify the nucleosomes, followed by staining with Coomassie blue to visualize proteins. Gels were then dried and the <sup>35</sup>S-methionine-labelled centromere proteins were visualized and quantified with a phosphorimager.

**CENP-N mutagenesis and stable cell lines.** Point mutants in CENP-N were generated with a site-directed mutagenesis kit (Stratagene) according to the

manufacturer's instructions. Stable cell lines were created by co-transfecting HEK293 flp-in target cells (Invitrogen) with pOG44 and a modified version of pcDNA5/FRT engineered to express CENP-N with an N-terminal GFP-tag according to the manufacturer's instructions. For western blotting and co-immunoprecipitation experiments, nuclei were isolated from ~5 $\times$ 10<sup>7</sup> cells and chromatin was solubilized with micrococcal nuclease as described previously<sup>5,8</sup>. After micrococcal nuclease treatment, extracts were centrifuged at 16,000g for 10 min in a microfuge and the concentration of each supernatant was determined using Bradford reagent (Biorad). The supernatant (10  $\mu$ g) was loaded on a 12.5% poly-acrylamide gel to determine the levels of the centromere proteins present (Fig. 2c) and the remaining extract (~2 mg) was used in anti-GFP immunoprecipitations. For CENP-A immunofluorescence, cells were fixed with methanol at -20 °C for 2 min without previous extraction and processed using standard techniques. For staining CCAN proteins, HEK293 cells were pre-extracted with 0.1% Triton X-100 in PBS for 1 min and then fixed for 10 min in PBS + 4% formaldehyde and processed using standard techniques. Similar methods were used for staining CCAN proteins in HeLa cells except that cells were pre-extracted for 3 min in 0.3% Triton X-100 before fixation.

**siRNA.** CENP-N (5'-GUAAAUUUCCGACAGAGAAUU-3', 5'-CUACCU-ACGUGGUGUACUAAUU-3', 5'-GAUAAUACCGAAAUGAAGAUU-3', 5'-CCAGAAAGUUUGGGAUGUUUU-3') and CENP-A (5'-AACACAGUCGG-CGGAGACAAG-3') siRNAs (Dharmacon) were used according to the manufacturer's instructions. Buffer alone or GAPDH siRNA was used in control experiments. Cells were processed for immunofluorescence as described above. For western blots, cell extracts were prepared by resuspending cells (20 mM HEPES, 0.5 M NaCl, 1% Triton X-100, 1 mM dithiothreitol, 10 mM EDTA, 10 mM EGTA and 1 mM PMSF), followed by centrifugation for 10 min at 16,000 g in a microfuge. Protein concentration in each extract was determined using Bradford reagent (Biorad) and identical amounts of total protein were loaded in each well of the gel.

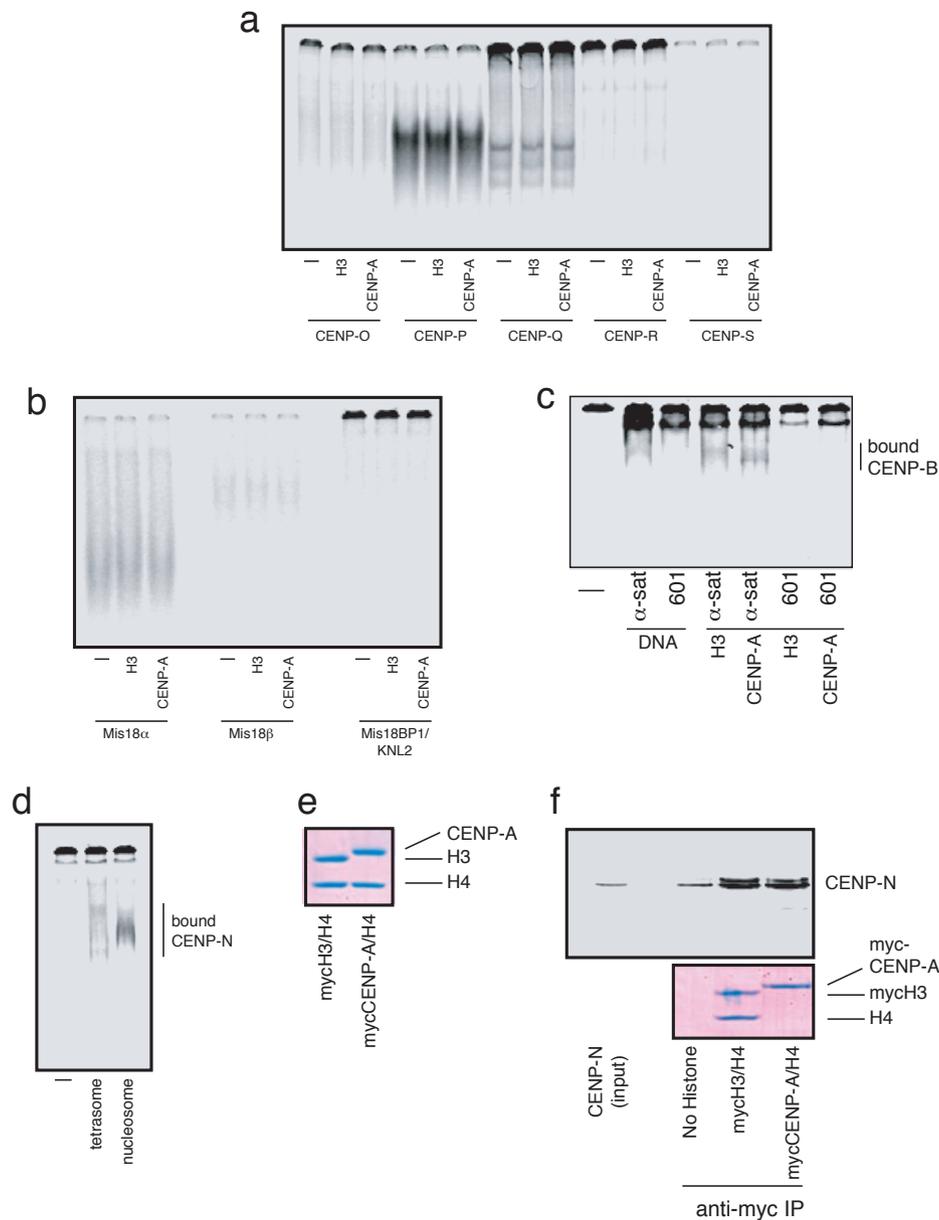
**SNAP pulse labelling.** HeLa cells stably expressing near endogenous levels of CENP-A-SNAP-3 $\times$ HA<sup>17</sup> were treated with thymidine (2 mM) for 17 h to arrest cells in S phase. Following release in deoxycytidine (24  $\mu$ M) for 3 h, cells were transfected with CENP-N and GAPDH siRNA pools (Dharmacon) according to the manufacturer's instructions. At 9 h after release from thymidine, fresh thymidine was added to synchronize cells at the next G1/S boundary. SNAP-tag was quenched with non-fluorescent BTP (Covalys) after which cells were released into S phase. Newly synthesized CENP-A-SNAP was labelled 7.5 h after release (in G2 phase) with TMR-Star (Covalys), cells were allowed to proceed through the cell cycle for CENP-A assembly and were collected at the next G1/S boundary by the addition of thymidine. Cells were fixed (without pre extraction) and processed for microscopy (see Supplementary Information, Fig. S5c for schematic).

**Microscopy.** Stacks of fixed cell immunofluorescence images encompassing the entire cell (or cells) were captured at 0.2  $\mu$ m axial steps using a motorized stage mounted on an Olympus IX70 microscope. Immunofluorescent images were acquired using a  $\times$ 60 1.4NA PlanApo objective and a CoolSnap-HQ CCD camera (Photometrics) on a DeltaVision Spectris system (Applied Precision). TMR-Star-labelled CENP-A-SNAP images were acquired using a  $\times$ 100 1.4 NA UPlanSApo objective and a Cascade2 EMCCD camera (Photometrics). Centromere fluorescence intensity in each channel was quantified as described except that intensity measurements were performed on non-deconvolved maximal intensity projections of each z-series<sup>24</sup>. The images presented are maximum intensity projections of deconvolved images.

23. Luger, K., Rechsteiner, T. J. & Richmond, T. J. Preparation of nucleosome core particle from recombinant histones. *Methods Enzymol.* **304**, 3–19 (1999).

24. Hoffman, D. B., Pearson, C. G., Yen, T. J., Howell, B. J. & Salmon, E. D. Microtubule-dependent changes in assembly of microtubule motor proteins and mitotic spindle checkpoint proteins at Ptk1 kinetochores. *Mol. Biol. Cell* **12**, 1995–2009 (2001).

DOI: 10.1038/ncb1899



**Figure S1** CENP-A nucleosome binding assay. **(a,b)** CENP-A or H3 nucleosomes or buffer alone (-) were incubated with the indicated centromere proteins and resolved on native gels as described in Figure 1c and 1d. **(c)** An experiment identical to that presented in Figure 2a was performed except that the nucleosome-binding requirement of CENP-B (1 nM) was determined. **(d)** An experiment identical to that in Figure 1a was performed with <sup>35</sup>S-labeled CENP-N (~10 nM) except that 100nM CENP-A/H4 tetrasomes (containing CENP-A/H4 and  $\alpha$ -satellite DNA) or

100 nM CENP-A nucleosomes were used. The control reaction (-) contained buffer only. **(e)** Purified myc-CENP-A/H4 and myc-H3/H4 tetramers used in anti-myc immunoprecipitations were resolved on a 17.5% gel and stained with coomassie blue. **(f)** Anti-myc immunoprecipitation of purified myc-CENP-A/H4 and myc-H3/H4 tetramers with <sup>35</sup>S-labeled CENP-N. Input represents 10% of total CENP-N in each immunoprecipitation. The myc-CENP-A/H4 tetramer (bottom panel) dissociates at the low salt concentrations used in these experiments.

a

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HsCENP-N -----MDETVAEFI*KRRTILKIPMNELTTILKAWDFLSENQLQTVNFR
BtCENP-N -----MDETLAEFFRRTILKIPMTEMMTILKTWNFMSENQLQTVNFR
MmCENP-N -----MKENVAEFLRRTILKIPLSEMKSILEAWDFLSEDLQLOTINLK
GgCENP-N -----MDEVIVEYIRRTVLKIPRDEIMAVLQKWGFLSEAQLQTVNFR
XlCENP-N MAPAAEIVQKRKRQASALVMDewLAEFIKRRTILKLPFSEtATILKtWGFtLteSeLQTFtLr

HsCENP-N *QRKESVVOHLIHLCEEKRASISDAALLDIYMQFHQHQKVVWDFVQMSKGPgedVDLFDmK
BtCENP-N QRKESIVQDLVLLCEENHASLNDAALHLDIYTFQFHRHQKIWDVDFQMSKAPGDDIDLFDME
MmCENP-N QRKDYLAQEVILLCEDKRASLDDVLLDIVYTFQFHRHQKLWNVDFQMSKEPgedVDLFDME
GgCENP-N QTKEGISHSVAQLCEESSADLKQAALLDIYNHIYPNKRVSVYHMKNT-GEETDFDFR
XlCENP-N YPKEVTATEVVRFCeARNATLDHAAALDLVFNHAYSNKKtWtVtYQMSKRleSenDlFDAS

HsCENP-N QFKNSFKKILQRALKNVTVSFRTEENAVWIRIAWGTOYTKPNQYKPTVYVYYSQTPYAF
BtCENP-N QFKSSFKKILQRALKNVTVSFRDAEENSvWIRIAWGTOYKkPNQYKPAyVYVYYSQTPYAF
MmCENP-N QFQSSFKRILQRALKNVTVSFRVYEKDSVWIRVAVGTOYSQPNQYKPTFVYVYYPQTPYAF
GgCENP-N DFKKKFRRQIQSALINVTINFREYEDNAIWIIRIAWGTPYTKPNQYKTSYVYVYHSQTPYVF
XlCENP-N EFKLQFKKSIHAVSKNVTINFKEFG-EALWIRIAWGTHNSRPNQYKATFAVYHSQTPYVF

HsCENP-N TSSSM LRRNTPLLGOALTIASKHHQIVKMDLRSRYLDSLKAIVFKQYNQTFETHNSTTPL
BtCENP-N TSSSR LKSNLPLLGOALTVAASKHHQIVKMDLRSRYLDSLKAIVFKQYNQSFETHNCTTSL
MmCENP-N ISSCHLKNTPLLHQALKVASKHHQIVHLDLRSRHLDLKAIVFREYNQTCENYSSTTSL
GgCENP-N ISASVLRSNLPLLCOAMVVASNYHDIHemELRSHCLNSLKDIVFKRYSQNFOTN---YPL
XlCENP-N ITG-LGKACQPLMCOALVIASKYSQIQEMELKSRCLeSLKDIvFKRFNqPFSSSHSKPHE

HsCENP-N QERSLGLDINMDSRIIHENIVEKERVORITQETFGDYPQPQLEFAQYKLETKFKSG-LNG
BtCENP-N QEGSLGLDINMDSRIIHENKVEKERVORVTOEIFGDYPQPRLEFAQYKLETKFKSD-LNG
MmCENP-N QEASL--SMCLDSKITHENTEEKVRVHRVTOETFGTYPOPQLEFAQYKLETKFKSN-IGG
GgCENP-N QERNV-LTENVDLRINDENRSEKERIYRLNQESFGNGPQPKLDFaQYKLETmFKSD-PKW
XlCENP-N KALTP---NIVDPRVTYENMREKDRVYHLtCETfGEGPLPKLELAsYKLETmFKADSAMS

HsCENP-N SILAErKEPLRCLIKFSSPHLLEALKSLAPAGIADAPLSPLLTcIPNkRMNYFKIRDK--
BtCENP-N GILAErEPLRCLVKFSSPHLLEALKSLAPAGIADAPLSPLLTcIPNkGKNYFKIRDK--
MmCENP-N GLLADrKEPFRCLVKFSSPHLLEALKSLAPAGIADAPLSPLLTcIPSkKNYFKIRDK--
GgCENP-N DVLE-kKEPFRCLVKFSSPHLLESkSLAPAGLADAPLSPLLTcIPQkARNYFKIREKKS
XlCENP-N GNLTAVNEPFRcVVKFSSPHLLEAIRSLAPAGIAEAPISLTLlScIPhKARNSFKITeKRS

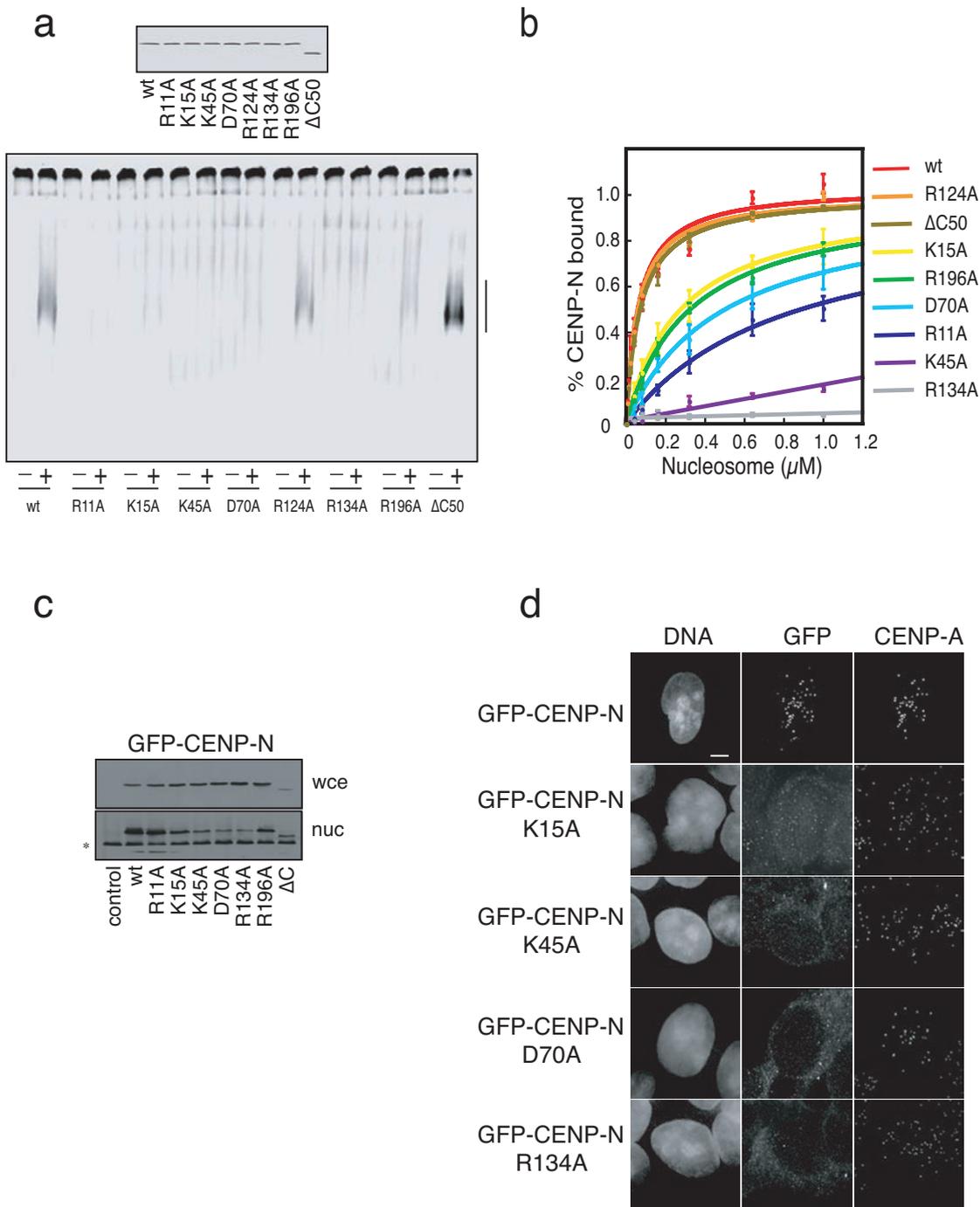
HsCENP-N -----
BtCENP-N -----
MmCENP-N -----
GgCENP-N LHPGSFVSP--
XlCENP-N MHPSSSQPTNV
    
```

b

	% Identity to HsCENP-N	
	1-289	290-339
BtCENP-N	84	93
MmCENP-N	70	93
GgCENP-N	56	83
XlCENP-N	70	68

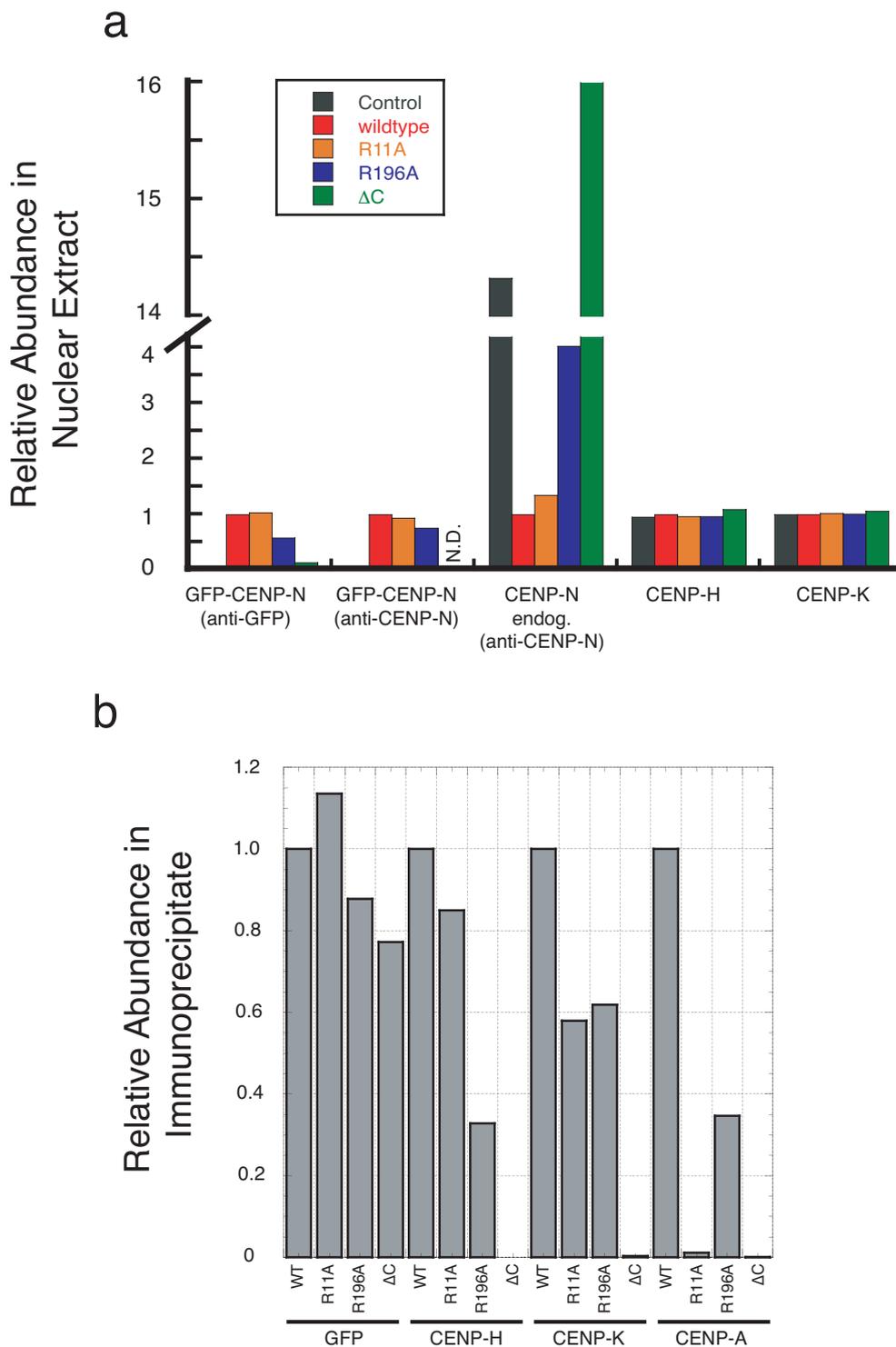
**Figure S2** Alignment of CENP-N orthologs. (a) CENP-N orthologs from several vertebrate species were aligned using Clustal W. Residues identical to those in human CENP-N are shaded in grey. Residues that were mutated to alanine in human CENP-N are indicated (\*). The residues

deleted in the CENP-NΔC truncation mutant are outlined in blue. (b) Comparison of the percent identity between human CENP-N and other vertebrate CENP-N orthologs over amino acids 1-289 or 290-339 of human CENP-N.



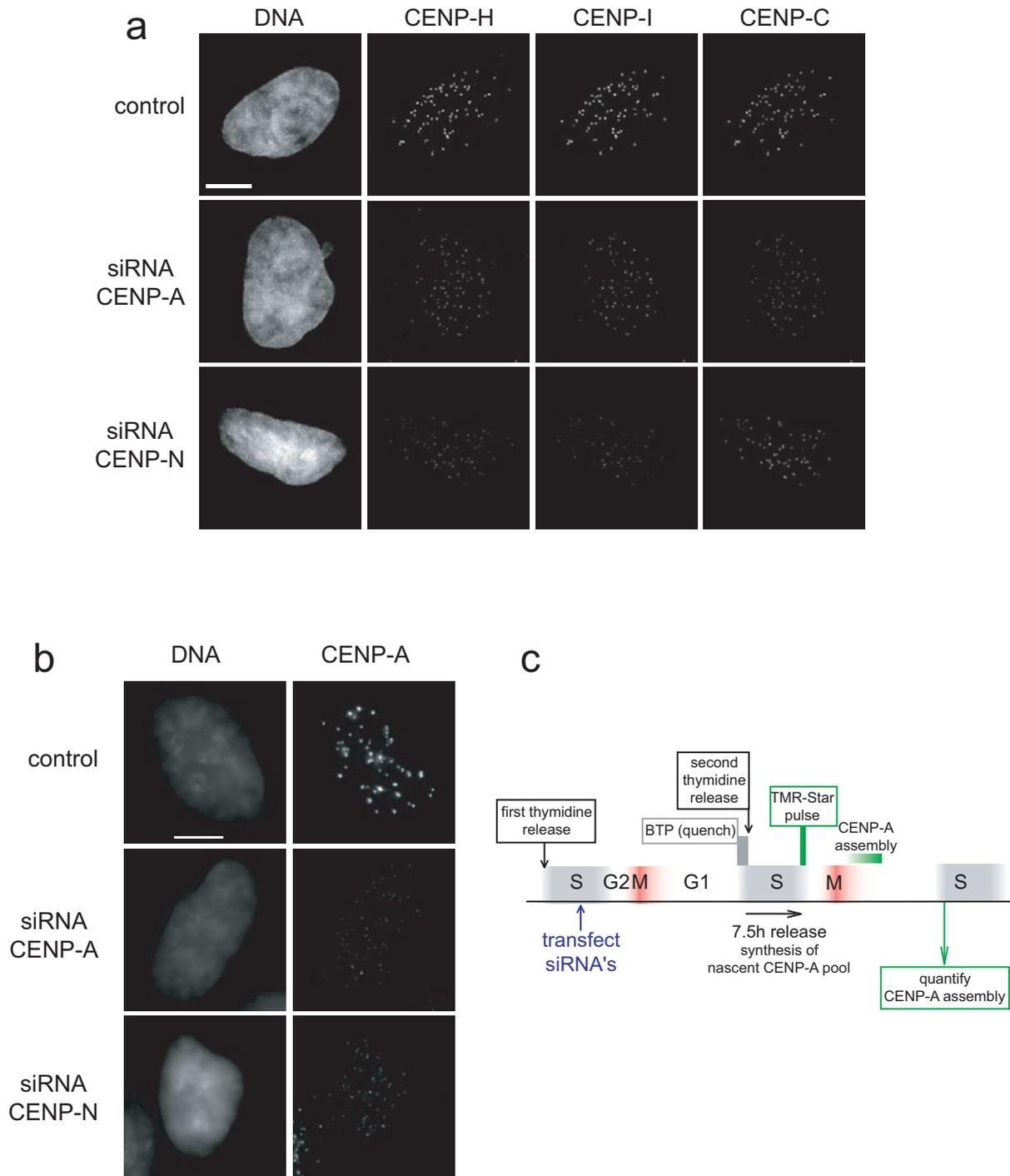
**Figure S3** Initial characterization CENP-N point mutants. **(a)** CENP-N mutants exhibit a range of affinities for CENP-A nucleosomes. CENP-N (wt) or individual CENP-N mutants were expressed in reticulocyte extract and resolved by SDS-PAGE (top panel). Each mutant was assayed for its ability to bind CENP-A nucleosomes (bottom panel). Nucleosome gel shift assays included wildtype CENP-N and each CENP-N mutant alone (-) or in the presence of 300 nM CENP-A nucleosome (+). **(b)** Dose-response experiments for each CENP-N mutant were performed as in A (bottom panel) with increasing concentrations of CENP-A nucleosome added to each reaction (N=3, error bars represent SEM). Data for wildtype CENP-N,

and the R11A, R196A and ΔC mutants is reproduced from Figure 2b for comparison. **(c)** Expression of CENP-N mutants in HEK293 cells. Whole-cell extracts (wce) or nuclear extracts (nuc) from stable HEK293 cell lines expressing GFP-CENP-N (wt) or the indicated GFP-CENP-N mutant were separated by SDS-PAGE and western blotted with anti-GFP antibodies. A nonspecific band (\*) that migrates faster than GFP-CENP-N is present in the nuclear extracts. **(d)** Representative images of HEK293 stable cells expressing GFP-CENP-N or the indicated GFP-CENP-N mutant. The image of cells expressing GFP-CENP-N is reproduced from Figure 3a and is included for reference.



**Figure S4** CENP-N mutants show changes in CENP-A nucleosome interaction and CCAN protein association. **(a)** Quantification of the levels GFP-CENP-N, endogenous CENP-N, CENP-H and CENP-K proteins from the western blots shown in Figure 2c. The amount of protein in each case is normalized to the

amount of protein present in the GFP-CENP-N lane. **(b)** Quantification of GFP-CENP-N wildtype and mutant immunoprecipitations from western blot in Figure 2d. The amount of protein in each case is normalized to the amount present in the GFP-CENP-N wildtype immunoprecipitation.



**Figure S5** CENP-N is required for centromere assembly. **(a,b)** Representative images of control cells or cells depleted of CENP-A or CENP-N with siRNA stained with the indicated antibodies to visualize centromere proteins. Scale

bar is 5  $\mu$ m. **(c)** Experimental scheme for SNAP based CENP-A assembly assay depicting order of synchronization, transfection and SNAP labeling steps described in detail in Material and Methods.

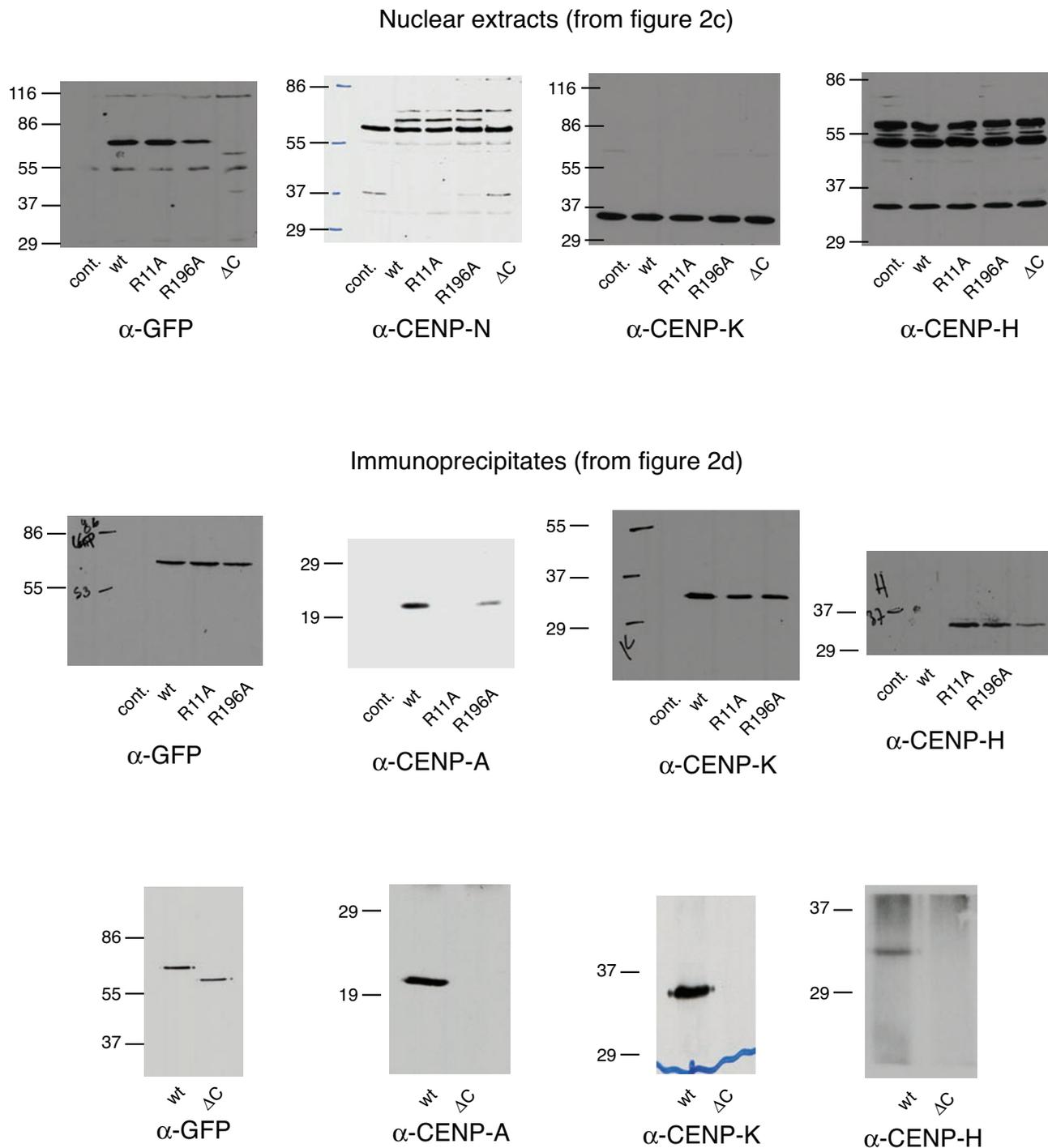


Figure S6 Full scans of western blot images.

CENP-N	$K_d$ apparent (nM)
wt	169±70
R11A	962±259
K15A	297±77
K45A	ND
D70A	546±152
R124A	72±10
R134A	ND
R196A	331±36
ΔC	62±8

**Table S1** The apparent dissociation constant of wild-type CENP-N and each CENP-N mutant for reconstituted CENP-A nucleosomes as determined by the dose-response experiments presented in Figure 2b and S5b. Affinities for R134A and K45A were too low to accurately determine a dissociation constant (ND).