SUPPLEMENTAL FIGURES AND LEGENDS

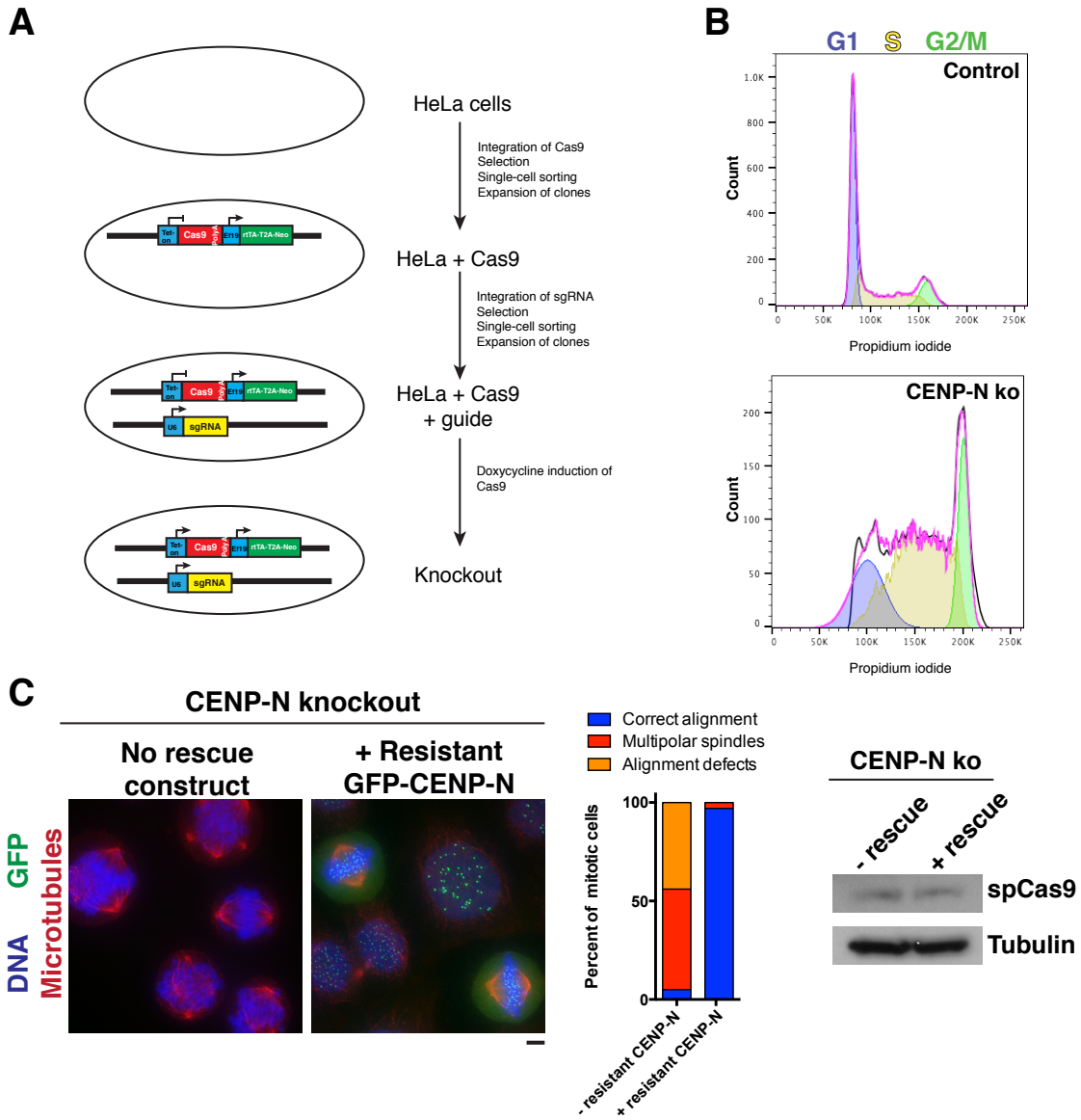

Figure S1. Generation of on-target inducible knockouts. Related to Figure 1. A) Flowchart for the generation of inducible knockouts using the CRISPR/Cas9 strategy. B) Representative analysis of DNA content of control cells (top) and CENP-N knockout cells (bottom) analyzed by flow cytometry after four days of Cas9 induction. Experimental data are shown in magenta, with G1/S/G2 populations that best fit the data based on the Watson Pragmatic model shown in blue, yellow and green. C) Rescue of the inducible CENP-N knockout by exogenous expression of GFP-CENP-N containing a mutated sgRNA targeting site. Left: immunofluorescence images of inducible CENP-N knockout cells, with or without the resistant CENP-N construct, induced for four days. Middle: quantification of mitotic phenotypes following four-day induction of the CENP-N knockout. Right: Western blot showing equivalent induction of spCas9 in both cell lines. Scale bar, 5 μm.

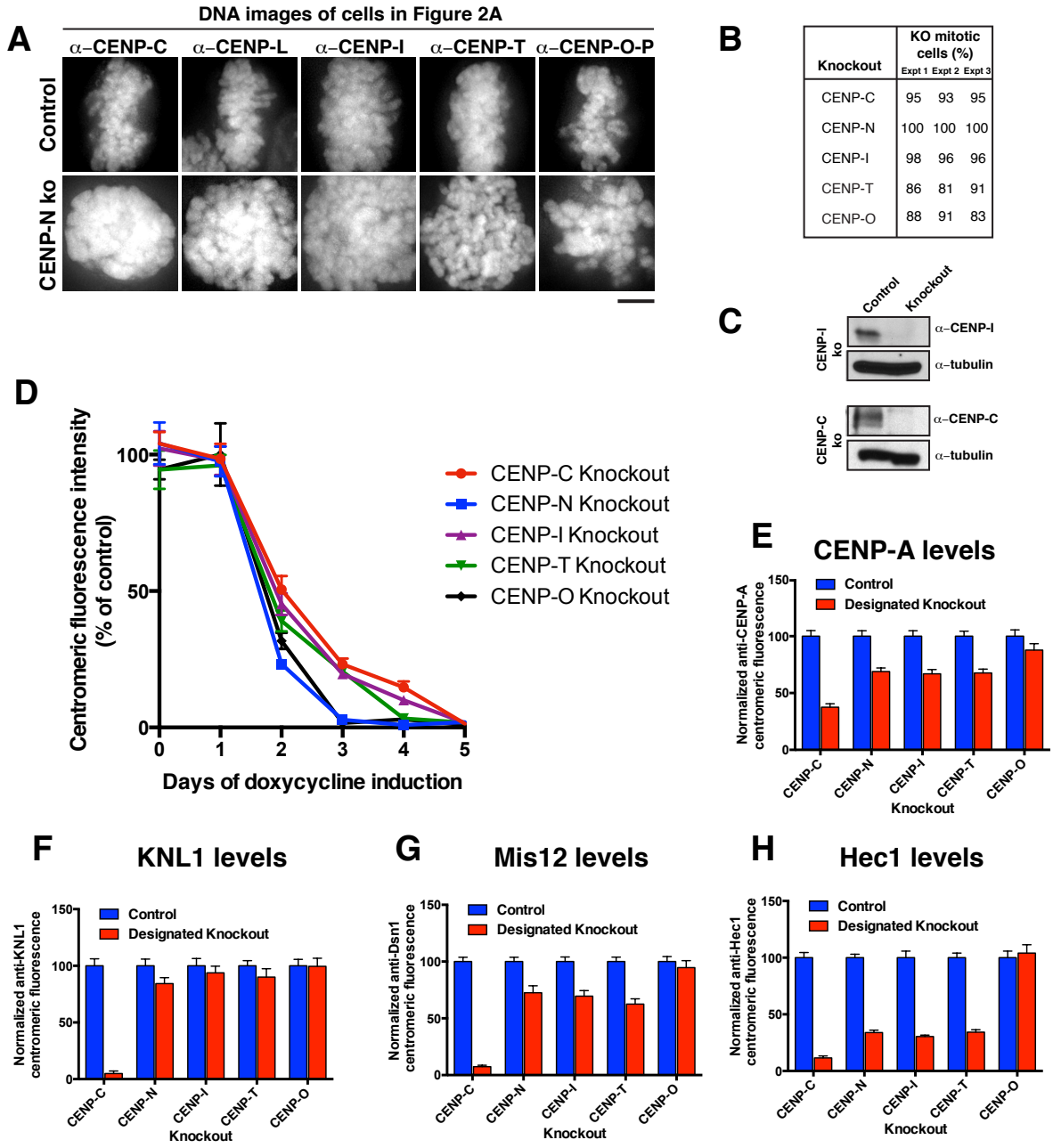

Figure S2. Functional relationships between kinetochore proteins. Related to Figure 2. A) Corresponding images of the DNA for the cells shown in Fig. 2A. B) Quantification of the proportion of mitotic cells in the population showing robust elimination of the sub-complex targeted by the knockout after 3 days of Cas9 induction from three independent experiments. We assessed the penetrance of the knockouts among mitotic cells, as these cells were tested for our quantitative immunofluorescence analysis. However, we note that mitotic cells are enriched in the knockouts over the interphase population due to the mitotic delay induced by most of the knockouts. C) Western blot analysis showing the absence of CENP-C and CENP-I in mitotic cells following induction of their respective knockouts for 5 days. D) Quantification of levels of the proteins corresponding to the genes targeted by the knockout over five days

following Cas9 induction (i.e. CENP-C knockout stained for α-CENP-C, etc. For the CENP-N knockout, α-CENP-L levels were analyzed). The fluorescence intensity of each antibody at centromeres was analyzed by quantitative immunofluorescence as in Fig. 2 and as described in the Experimental Procedures. Points represent the mean centromeric fluorescent intensity of a given antibody in the knockout as percentage of the fluorescent intensity of the antibody in control cells. Error bars represent s.e.m, n = 20 cells per condition per antibody per knockout per time point. E-H) Mean centromeric fluorescence intensity of anti-CENP-A (E), anti-KNL1 (F), anti-Dsn1 (G) or anti-Hec1 (H) in mitotic cells following induction of the indicated knockouts for five days, normalized to cells in which spCas9 is not induced. n = 20 cells per condition per antibody per knockout, error bars represent s.e.m. Note that these data are represented orthogonally to the data in Figure 2. In Fig. 2, each graph depicts the levels of all CCAN components in a single knockout. Here, each graph depicts the levels of a single component in all of the knockouts.

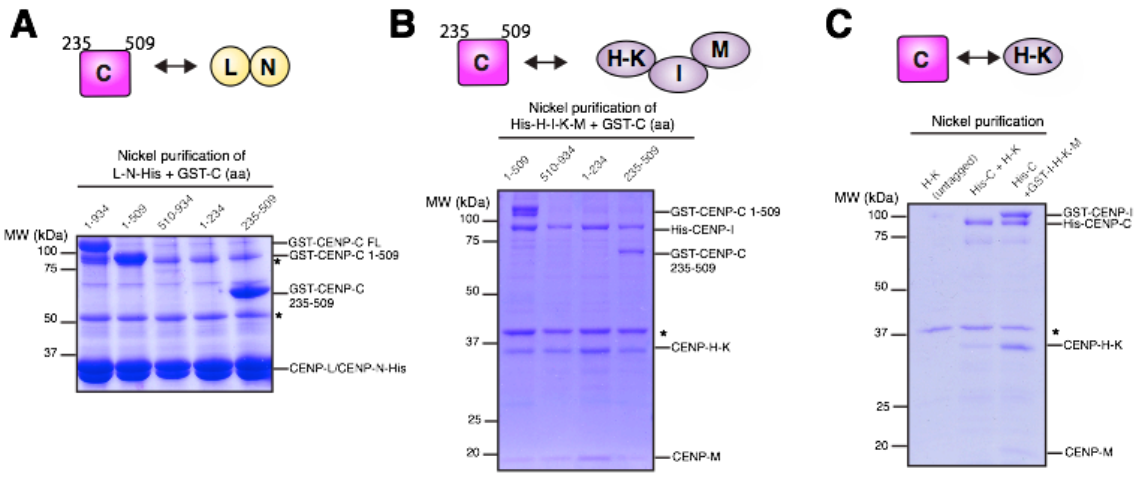

Figure S3. Interactions of CENP-C, CENP-L-N and CENP-H-I-K-M. Related to Figure 3. A) SDS-PAGE gel showing co-purification of fragments of GST-CENP-C with CENP-L-N-His. B) SDS-PAGE gel showing co-purification of fragments of GST-CENP-C with His-CENP-I-H-K-M. C) SDS-PAGE gel showing Nickel purification of His-CENP-C with untagged CENP-H-K. Gels were stained with Coomassie Brilliant Blue. * : contaminant.

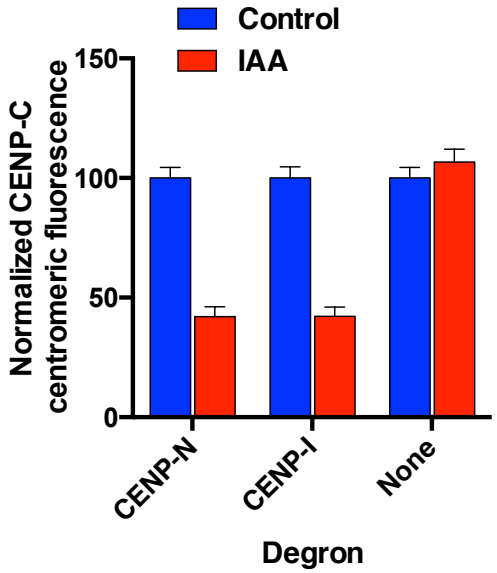

Figure S4. Effects of auxin-hormone treatment on CENP-C levels. Related to Figure 4. Quantification of centromeric anti-CENP-C levels in interphase cells from an asynchronous population 5 h following the addition of IAA. Parental DLD-1 cells lacking any AID-tagged alleles do not show reduction in CENP-C levels upon IAA treatment.

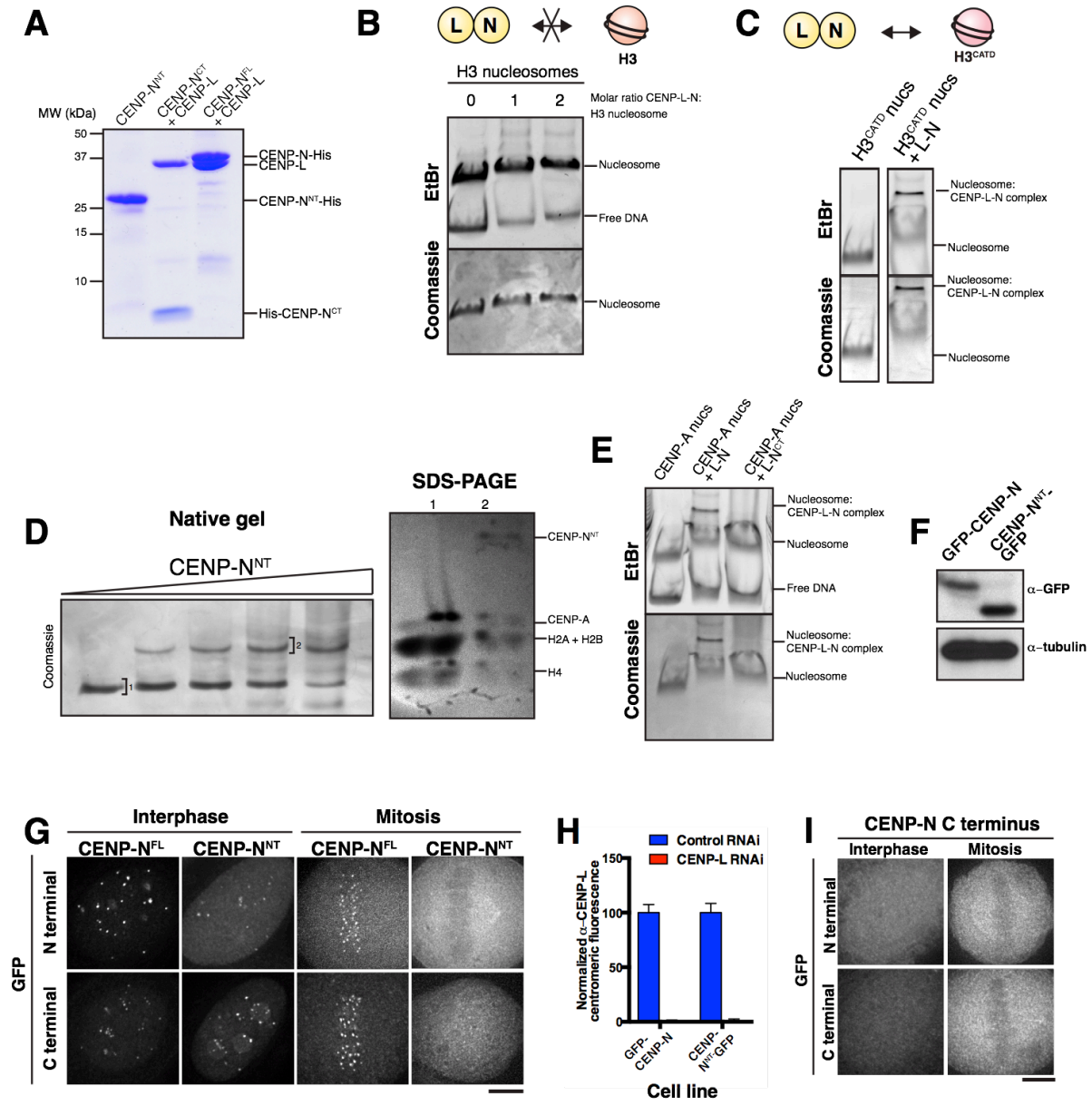

Figure S5. Interactions of CENP-L-N at the nucleosome interface. Related to Figure 6. A) SDS-PAGE gel showing the purification of CENP-N^{NT}, CENP-L-N^{CT}, and full-length CENP-L-N. B) Native gel assessing binding of 145 bp histone H3-containing nucleosomes to increasing concentrations of CENP-L-N. C) Native gel showing interactions of CENP-L-N with 145 bp $H3^{CATD}$ chimeric nucleosomes. D) 2-dimensional gel analysis of binding of CENP-N^{NT} to CENP-A nucleosomes. Left: native gel of CENP-A nucleosomes with increasing amounts of CENP-N^{NT}. The bands marked 1 and 2 were excised and run on an SDS-PAGE gel (right) to confirm the species (CENP-A nucleosomes and/or CENP-N^{NT}) in the native gel band. E) Native gel showing failure of CENP-L-N^{CT} to bind to CENP-A nucleosomes. F) Western blot testing levels of the GFP-CENP-N and CENP-N^{NT}-GFP transgenes in stable cell lines used in Fig. 6D and E. G) Immunofluorescence images showing interphase and mitotic localization of transiently transfected CENP-N constructs tagged with GFP at the N- or C-terminus.

Images are not scaled equivalently, but are scaled to show the full range of data. CENP-N^{NT} does not localize to kinetochores when tagged N-terminally, and localizes very weakly to kinetochores and diffusely to bulk chromatin when tagged C-terminally. This localization is more apparent in live cells, as shown in Fig. 6D. H) Mean centromeric fluorescence intensity of α-CENP-L in cells expressing GFP-CENP-N or CENP-N^{NT}-GFP, depleted for CENP-L by RNAi. Error bars represent s.e.m, n = 20 cells per condition. I) Immunofluorescence images of cells in interphase and mitosis following transient transfection of CENP-N^{CT} tagged with GFP at the N- or C-terminus. Gels were stained with Ethidium Bromide (EtBr) and Coomassie Brilliant Blue as indicated. Scale bars: 5 μm.

Table S1. Cell lines used in this study. Related to Experimental Procedures.

Name	Description of transgene	Expression	Background	Source
HeLa	-			Cheeseman lab
U2OS-lacO	lacO array		U2OS	Susan Janicki (Janicki et al., 2004)
DLD-TIR1	os-TIR1-9x myc	Constitutive	DLD-1	Andrew Holland (Holland et al., 2012)
cTT20	tetOn-Cas9	Inducible	HeLa	This study
cKM149	CENP-I-AID-EGFP	Endogenous	DLD-1-TIR1	This study
cKM153	CENP-C knockout	Inducible	cTT20	This study
cKM154	CENP-N knockout	Inducible	cTT20	This study
cKM157	CENP-I knockout	Inducible	cTT20	This study
cKM202	CENP-N-AID-EGFP	Endogenous	DLD-1-TIR1	This study
cKM207	CENP-T knockout	Inducible	cTT20	This study
cKM209	CENP-O knockout	Inducible	cTT20	This study
cKM204	GFP-CENP-N CRISPR resistant	Constitutive	cKM154	This study
cKM227	GFP-CENP-N (1-240) CRISPR resistant	Constitutive	cKM154	This study
cKM230	GFP-CENP-N (1-240) CRISPR resistant	Constitutive	cKM153	This study

Table S2. Antibodies used in this study. Related to Experimental Procedures.

Antigen	Antibody	Source
Tubulin	Mouse anti-tubulin (DM1α)	Sigma
Human centromere proteins	Human anti-centromere antibodies (ACA)	Antibodies Inc.
CENP-A	Mouse anti-CENP-A (3-19)	Abcam
CENP-C	Rabbit anti-CENP-C N-terminus	Cheeseman lab (Gascoigne et al., 2011)
CENP-L	Rabbit anti-CENP-L	This study
CENP-T	Rabbit anti-CENP-T	Cheeseman lab (Gascoigne et al., 2011)
CENP-I (IF)	Rabbit anti-CENP-I	Tim Yen (Liu et al., 2006)
CENP-I (Western)	Rabbit anti-CENP-I	Abcam
CENP-K	Rabbit anti-CENP-K	This study
CENP-O-P	Rabbit anti-CENP-O and CENP-P	This study
Hec1	Mouse anti-Hec1 [9G3]	Abcam
KNL1	Rabbit anti-KNL1	Cheeseman lab (Cheeseman et al., 2008)
Dsn1	Rabbit anti-Dsn1	Cheeseman lab (Kline et al., 2006)
GFP	Rabbit anti-GFP	Cheeseman lab
Tubulin	Mouse anti-tubulin (Dm1α)	Sigma

Table S3. Oligonucleotides used in this study. Related to Experimental Procedures.

Purpose	Sequence (5'-3')
CENP-C knockout	CACCGAGAGCACTGCACTCCTTCA AAACTGAAGGAGTGCAGTGCTCTC
CENP-I knockout	CACCGAGAACGTCCAGGCACAAAAC AAACGTTTTGTGCCTGGACGTTCTC
CENP-N knockout	CACCGCCAGTACAAACCTACCTACG AAACCGTAGGTAGGTTTGTACTGGC
CENP-T knockout	CACCGCAGCGGACCCGCGCACCCCG AAACCGGGGTGCGCGGGTCCGCTGC
CENP-O knockout	CACCGTTTACGGGATCTGCTCACT AAACAGTGAGCAGATCCCGTAAAC
CENP-I C-terminal tag sgRNA	CACCGTCCTCAAGGAGTACTCAGAC AAACGTCTGAGTACTCCTTGAGGAC
CENP-N C-terminal tag sgRNA	CACCGCCTGCTAACTGTAGCCGTTG AAACCAACGGCTACAGTTAGCAGGC
CENP-I C-terminal tag 5' homology arm	GCGCGGGGCCCCATTCCAGTGACTTTAATGTGAATTTA GCGCGGTCGACATATTGATTGTTGCAGTTTATGCC
CENP-I C-terminal tag 3' homology arm	GCGCGGGATCCATGAATGTTGACATAAACTGAACAC GCGCGCCGCGGTTCTGAAAGTAAAATTTCAGTTAGTTTA TTTAGT
CENP-N C-terminal tag 5' homology arm	GCGCGGGCCCGGCACTGTGCATGCTGGCATT GCGCGTCGACtttatctctaattttaaaataattcattctcttgttggg
CENP-N C-terminal tag 3' homology arm	Synthesized by Genewiz, bookended by the following: GACGTGCGTGGTTTCTT CTGCACCACCATGCCCAGC

Table S4. Bacmids used in this study. Related to Experimental Procedures.

Name	Description of bacmid
pKM394	CENP-L; CENP-N-His
pKM377	CENP-H; CENP-K; CENP-M; His-CENP-I
pKM376	CENP-H; CENP-K; CENP-M; GST-CENP-I
pKM197	His-CENP-C
pKM568	GST-CENP-C
pKM672	GST-CENP-C 1-509
pKM673	GST-CENP-C 510-934
pKM691	GST-CENP-C 1-234
pKM692	GST-CENP-C 235-509
pKM498	His-CENP-T; CENP-W
pKM410	GST-CENP-T; CENP-W
pKM652	His-CENP-T; CENP-W; CENP-S; GST-CENP-X
pSAF60	CENP-H; CENP-K (untagged)
pDK580	CENP-L; His-CENP-N
pDK611	CENP-L; His-CENP-N 241-339
pKM634	CENP-I; CENP-H; CENP-K; CENP-M (all untagged)
pKM499	His-CENP-U; CENP-O; CENP-P; CENP-Q
pKM610	CENP-R (untagged)
pKM744	His-CENP-T; CENP-W$^{\text{DNA binding mutant}}$ (R19A, K23A, R24A, R34A, R87A; (Nishino et al., 2012).

Table S5. Buffers used in this study. Related to Experimental Procedures.

Name	Contents
His lysis buffer	50 mM NaPi pH 8.0 300 mM NaCl 10 mM imidazole pH 8.0 0.1 % Tween-20
His wash buffer 300	50 mM NaPi pH 8.0 300 mM NaCl 40 mM imidazole pH 8.0 0.1 % Tween-20
His wash buffer 500	50 mM NaPi pH 8.0 500 mM NaCl 40 mM imidazole pH 8.0 0.1 % Tween-20
His elution buffer 300	50 mM NaPi pH 8.0 300 mM NaCl 250 mM imidazole pH 8.0
His elution buffer 500	50 mM NaPi pH 8.0 500 mM NaCl 250 mM imidazole pH 8.0
GST wash buffer 250	50 mM NaPi pH 8.0 250 mM NaCl 0.1 % Tween-20
GST wash buffer 500	50 mM NaPi pH 8.0 500 mM NaCl 0.1 % Tween-20
GST elution buffer	50 mM Tris pH 8.0 10 mM reduced glutathione 75 mM KCl

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Quantification of centromeric fluorescence intensity

Quantification of fluorescence intensity was conducted on unprocessed images using Metamorph (Molecular Devices). Cells in either prometaphase or metaphase (or with chromosome alignment defects, but condensed chromosomes) were quantified. A region-of-interest of fixed size was placed over each kinetochore (as defined by CENP-A staining) and the integrated antibody fluorescence intensity of each region-of-interest was measured and corrected for local background. The mean intensity for the visible kinetochores of a given cell was then calculated. The procedure was repeated for each cell.

Generation of the AID-EGFP repair template

To generate the repair plasmid for the auxin degron-tagged alleles, the AID-EGFP sequence from pCDNA5-AID-EGFP was amplified and inserted in place of eYFP in the donor plasmid described previously (McKinley and Cheeseman, 2014). Briefly, this plasmid contains a 9-residue linker (as in pCDNA5-AID-EGFP), the AID-EGFP sequence, and the neomycin resistance gene under control of the PGK promoter. For each target, a DNA sequence of ~1 kb up to, but not including, the stop codon of the gene of interest (the 5' homology arm) was amplified from HeLa genomic DNA by PCR using the oligonucleotide sequences in Table S3 and cloned upstream and in frame with the AID-EGFP sequence. A DNA sequence of ~1 kb immediately following the stop codon of the gene of interest (the 3' homology arm) was then amplified and cloned downstream of the neomycin resistance gene. To prevent cutting of the EGFP-AID-

tagged allele by SpCas9, the repair plasmid was then mutated to disrupt the PAM site in the 3' homology arm.

Protein purification

The buffers used for protein purification are listed in Table S5. All insect cell pellets were drop-frozen in His lysis buffer in liquid nitrogen and thawed in His lysis buffer with 1 mini-EDTA-free protease inhibitor tablet (Roche) and PMSF. Lysates were sonicated and centrifuged at 40,000 x g for 30 mins and the cleared supernatant bound to Ni-agarose (Qiagen) or glutathione-agarose (Sigma) for 1 h, washed three times in His or GST wash buffer and eluted in His or GST elution buffer. For co-purification of insect cell complexes with CENP-N^{NT}-His from bacteria, bacterial pellets were frozen in His lysis buffer at -80 ° C, thawed and lysed using 1 mg/ml lysozyme and centrifuged at 40,000 x g for 30 mins. The insect and bacterial supernatants were then combined in the presence of Ni-NTA agarose beads, washed three times and eluted as for complexes co-expressed in insect cells (above). All complexes co-expressed on a single bacmid were purified in 500 mM NaCl buffers. For co-infections, the following buffers were used: CENP-C/CENP-L-N complexes and CENP-L-N/CENP-H-I-K-M complexes were purified in 500 mM NaCl buffers. All other co-purifications or tests of interactions were performed in 300 mM NaCl buffers for the Nickel prep, and 250 mM NaCl buffers for the GST prep.

SUPPLEMENTAL REFERENCES

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