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Supplemental Experimental Procedure

Transfection for RNAi and plasmid

RNAi experiments were conducted using LipofectamineRNAi MAX (Invitrogen) using the manufacturers instructions. siRNA transfections were performed with a total of 100 nM of siRNA duplex and siRNA sequences of CENP-T, CENP-C, CENP-Q, and CENP-H described previously (Gascoigne et al., 2011; Okada et al., 2006). Transient transfection of cells with plasmids or establishment of stable cell encoding GFP fusion proteins: GFP-CENP-N, CENP-N-GFP, CENP-R-GFP, GFP-CENP-H, CENP-H-GFP, GFP-CENP-M, CENP-M-GFP, GFP-CENP-K, CENP-K-GFP, CENP-O-GFP, GFP-CENP-P, CENP-P-GFP, GFP-CENP-C426 (426-943 aa), GFP-CENP-C690 (690-943 aa), GFP-CENP-C759 (759-943 aa), GFP-CENP-T107 (107-561 aa), GFP-CENP-T, CENP-T-GFP. GFP-CENP-C, and CENP-C-GFP were performed with transfection reagent (Effectene; QIAGEN) according to the manufacturer's instructions. We analyzed only cells expressing lower levels of GFP fusion proteins. For stable cell lines, we added Neomycin (Gibco) after transfection and selected cell lines by Western blot expressing similar levels as endogenous proteins. For all CENP-T and CENP-C GFP fusion protein constructs, we used an RNAi-resistance sequence. cDNA of CENP-R, CENP-N, CENP-H, CENP-C and CENP-T were a gift from Dr. IM Cheeseman (Whitehead Institute, MIT, USA). GFP fusion to CENP-C or CENP-T rescued the depletion of endogenous protein by siRNA by several criteria. The depletion of CENP-C or CENP-T induced around 60% reduction of Ndc80/Hec1, but GFP-fusion proteins recovered control level of Ndc80 recruitment to the kinetochore. In addition, the mean separation between CENP-A and Ndc80/Hec1(9G3) increased in CENP-C or CENP-T depletion (Figure 3A), and this

phenotype was also rescued by GFP-fusion protein (Figure 2A and Figure S3C-D).

Immunofluorescence Labeling

Sources of Primary Antibodies and Their Antigens

Antibody	Antigen	Source	Reference
Rabbit anti-CENP-A	Full length	Aaron Straight	Wan et al., 2009
Rabbit anti-Spc24	Full length	Todd Stukenberg	Varma et al., 2013
Rabbit anti-CENP-I	N-term 249	Tim Yen	Liu et al., 2003
Rabbit anti-CENP-C	Full length	Iain Cheeseman	Gascoigne et al., 2011
Rabbit anti-CENP-T	Full length	Iain Cheeseman	Gascoigne et al., 2011
Mouse anti-Hec1 (9G3)	200-222	Abcam	Wan et al., 2009
Rabbit anti-CENP-Q	Full length	Abcam	This paper
Rabbit anti-CENP-H	1-50	Bethyl	This paper
Rabbit anti-CENP-K	Full length	MBL	This paper
Rabbit anti-CENP-50/U	Full length	MBL	This paper
Rabbit anti-GFP	GFP	Invitrogen	This paper
Mouse anti-GFP	GFP	Abcam	This paper
Rabbit anti-Ser44-P	Ser44-P (Hec1)	Jennifer DeLuca	DeLuca et al., 2011
Rabbit anti-Ser55-P	Ser55-P (Hec1)	Jennifer DeLuca	DeLuca et al., 2011
Rabbit anti-CENP-I	700-756	Abcam	This paper
Rabbit anti-phospho Knl1	Ser 24-P	Iain Cheeseman	Welburn et al., 2010

Imaging

For image acquisition, 50 frame 3D stacks of pairs of red and green fluorescent images were obtained sequentially at 200 nm steps along the z axis through the cell using MetaMorph 6.1 software (Molecular Devices) and a high-resolution Nikon TE300 inverted microscope equipped with a Yokogawa CSU10 spinning disk confocal with image magnification yielding a 65 nm pixel size from the Orca ER cooled CCD camera (Wan et al., 2009) and an 100X/1.4NA (Planapo) DIC oil immersion objective (Nikon).

Fluorescence Intensity Measurement

The Z-axis position of best focus (maximum integrated intensity) was used for

analysis of kinetochore fluorescence. A 9 x 9 pixel region was centered on the fluorescent kinetochore spot to obtain integrated fluorescence, while a 11x11 pixel region centered on the 9 x 9 pixel region was used to obtain surrounding BG intensity. Fi (integrated kinetochore fluorescence minus BG) = integrated intensity for 9 x 9 region – (integrated counts for the 11 x 11 region – integrated counts for 9 x 9 region) x pixel area of the 9 x 9 region / (pixel area of the 11 x 11 region – pixel area of a 9 x 9region) as described previously (Lawrimore et al., 2011). Measurements were made with Metamorph 7.7 analysis software (Molecular Devices) using the region measurements tool. For analysis of phospho-Hec1 (Ser44-P or Ser55-P) or kMT intensities, we normalized Fi of the phospho-Hec1 (Ser44-P or Ser55-P) or kMT by Fi of Ndc80/Hec1(9G3) intensities (total Hec1) from same kinetochore in a different fluorescent color.

Supplementary Figure legends

Supplementary Figure S1 (Related to Figure 1)

Delta analysis for control metaphase cells of the separation between the following CCAN proteins labels and Ndc80/Hec1(9G3) listed above each Delta plot (A-L).

Supplementary Figure S2 (Related to Figure 2)

CCAN proteins localize at peripheral surface of centromeric chromatin containing CENP-A at metaphase. (A-D) Two-color immunofluorescence (left) and Delta analysis (right) for the pairs of protein labels listed above each low magnification immunofluorescence image. Note, antibodies were used to label GFP. Mean Delta values are given on each plot and n = number of sister pairs measured.

Supplementary Figure S3 (Related to Figure 2)

The mean centroid position of CENP-C locates deeper within the inner centromere depending on amount of expression. (A) The schematic representation of human CENP-C. The color boxes indicate Central DNA binding domain (426-537) and the two Mif2 DNA binding domains (736-758 and 846-843). CC426 removes N-terminus region of CENP-C. CC690 removes N-terminus region and Central domain. CC759 removes N-terminus region, Central domain, and one of the Mif2 domains. (B) Two-color immunofluorescence using Ndc80/Hec1(9G3) and GFP antibodies for cells expressing GFP-CC426, GFP-CC690, and GFP-CC759. (C) Summary of kinetochore localization of CENP-C mutants. (D) Two-color immunofluorescence using CENP-A and GFP antibodies for cells with low (upper) and high (lower) expression of GFP-CC426. (E) The dependence between the mean separations of GFP-CC426 from

CENP-A at metaphase as a function of the expression level of GFP-CC426 measured by the integrated fluorescence intensity of immunofluorescently labeled GFP-CC426. (F) Two-color immunofluorescence using tubulin and GFP antibodies for cells with low (upper) and high (middle and lower) expression of GFP-CC426. (G) The DAPI images 48 hours after transfection of control (left) and GFP-CC426 (right).

Supplementary Figure S4 (Related to Figure 3)

The stretch of the Ndc80 complex does is not affected by depletion of CCAN complex for bi-oriented chromosomes in late prometaphase. (A) CENP-A is over incorporated at metaphase centromeres in the HeLa cell line stability expressing GFP-CENP-A that was used in the previous Delta analysis by Wan et al., 2009. Two-color immunofluorescence using CENP-A and GFP antibodies in control or GFP-CENP-A cells (left). Integrated fluorescence intensity at kinetochores normalized by the mean level measured for control cells (right). (B) Immunofluorescence images showing changes in kinetochore concentration of CCAN proteins relative to Ndc80/Hec1(9G3) and control cells for the proteins and RNAi indicated: CENP-Q, Ndc80/Hec1(9G3), and CENP-C in control cells or CENP-Q depleted cells (top, left); CENP-T, Nc80/Hec1(9G3), and CENP-C in control cells or CENP-C depleted cells (top, middle); CENP-T, Ndc80/Hec1(9G3) and CENP-C in control cells or CENP-T depleted cells (bottom, left); CENP-H, Ndc80/Hec1(9G3), and CENP-C in control cells or CENP-H depleted cells (bottom, middle); and CENP-H, Ndc8/Hec1(9G3) and CENP-C in control cells or CENP-C/CENP-H depleted cells (top, right). Measurement of Ndc80/Hec(9G3) and CCAN protein intensities normalized to their values in control cells for the RNAi treated cells (bottom, right). (C) Kinetochore immunofluorescence of both Spc24 and Ndc80/Hec1(9G3) in control cells, CENP-Q

RNAi cells, CENP-C RNAi cells, CENP-T RNAi cells, CENP-H RNAi cells or CENP-C/CENP-H RNAi cells (upper). Corresponding Delta analysis (lower). (D) The mean separation between Spc24 and Ndc80/Hec1(9G3) does not change between experimental and control cells in (C).

Supplementary Figure S5 (Related to Figure 5)

Criteria used to select kinetochores for Delta analysis based on compaction of CENP-A chromatin. Delta analysis was performed for kinetochores exhibiting CENP-A fluorescence where the thickness of the kinetochores along the kMT or K-K axis appeared less than or equal the width of the face of the kinetochore. For the quantification of the percentage of kinetochores with de-compacted CENP-A chromatin, line scans were obtained along the kMT axis through the central width of the kinetochores and perpendicular along the face of the kinetochore, and CENP-A chromatin was identified as de-compacted when the full width at half-maximal intensity along the kMT or K-K axis was greater than for the face of the kinetochore.

Supplementary Figure S6 (Related to Figure 6)

Hyper intra-kinetochore stretch is associated with a reduction in both the level of phosphorylation of Knl1 and the level of kinetochore Mad1 in late prometaphase. (A) Two-color immunofluorescence for Ndc80/Hec1(9G3) and phospho-Knl1 at kinetochores in control cells, CENP-C, or CENP-T depleted cells. (B) Integrated fluorescence intensity of phospho-Knl1 for bi-oriented (left) or not bi-oriented (right) kinetochores in control, CENP-C, or CENP-T depleted cells. (C) Two-color immunofluorescence for Ndc80/Hec1(9G3) and tubulin at kinetochores in control cells, CENP-C, or CENP-T depleted cells with cold treatment. (D) Integrated

fluorescence intensity of kMT at kinetochores normalized by the value for Ndc80/Hec1(9G3) on bi-oriented kinetochores in control, CENP-C, or CENP-T depleted cells. (E) Integrated fluorescence intensity of Mad1 at bi-oriented (left) or not bi-oriented (right) kinetochores in control late prometaphase, CENP-C, CENP-T, CENP-C/-H depleted cells. (Student' t-test * $p < 0.10$, ** $p < 0.05$)

	Distance (nm ± S.D.)	N number	K-K DIstance (nm ± S.D.)
CENP-50 (U)-Hec1 (9G3)	48.4 ± 8.6	n = 145	1265.3 ± 238.7
CENP-A-Hec1 (9G3)	84.5 ± 7.7	n = 127	1129.9 ± 209.7
CENP-H-Hec1 (9G3)	51.8 ± 6.5	n = 179	1516.8 ± 350.7
GFP-CENP-H-Hec1 (9G3)	54.7 ± 5.0	n = 103	1194.2 ± 209.9
CENP-H-GFP-Hec1 (9G3)	55.9 ± 8.2	n = 101	1444.2 ± 281.6
CENP-I-Hec1 (9G3)	58.6 ± 5.4	n = 105	1194.7 ± 197.7
CENP-I (C-term)-Hec1 (9G3)	41.4 ± 10.3	n = 204	1202.6 ± 179.3
CENP-K-Hec1 (9G3)	55.9 ± 7.0	n = 183	1196.1 ± 221.3
GFP-CENP-K-Hec1 (9G3)	63.3 ± 6.3	n = 84	1210.3 ± 223.5
GFP-CENP-N-CENP-A	22.6 ± 6.0	n = 107	1171.8 ± 214.2
CENP-K-GFP-Hec1 (9G3)	67.7 ± 8.2	n = 90	1522.2 ± 303.1
GFP-CENP-L-Hec1 (9G3)	64.2 ± 5.8	n = 103	1390.9 ± 336.2
CENP-L-GFP-Hec1 (9G3)	63.0 ± 9.0	n = 85	1354.1 ± 196.5
GFP-CENP-M-Hec1 (9G3)	59.9 ± 6.9	n = 96	1595.0 ± 378.1
CENP-M-GFP-Hec1 (9G3)	62.3 ± 7.5	n = 96	1314.7 ± 311.6
CENP-O-GFP-Hec1 (9G3)	66.0 ± 5.3	n = 93	1353.0 ± 256.1
GFP-CENP-P-Hec1 (9G3)	63.4 ± 7.0	n = 96	1500.8 ± 365.6
CENP-P-GFP-Hec1 (9G3)	65.6 ± 6.2	n = 96	1392.0 ± 246.2
CENP-Q-Hec1 (9G3)	63.4 ± 6.8	n = 110	1250.3 ± 220.7
CENP-R-GFP-Hec1 (9G3)	64.5 ± 5.9	n = 104	1339.1 ± 203.6
CENP-T (M)-Hec1 (9G3)	60.0 ± 5.8	n = 89	1234.3 ± 169.7
CENP-C (M1)-Hec1 (9G3)	60.6 ± 5.8	n = 66	1329.8 ± 265.3
CENP-C (M2)-Hec1 (9G3)	62.5 ± 4.6	n = 62	1365.8 ± 276.3

Table S1 (Related to Figure 1)

Summary of mean Delta values measured for separation between CCAN proteins labels and Ndc80/Hec1(9G3) at metaphase. Included are the number of kinetochore pairs analyzed (n), and mean sister kinetochore K-K separation of Hec1(9G3) labels for metaphase sister kinetochore pairs. Positive values are inward (towards the centromeric chromatin).

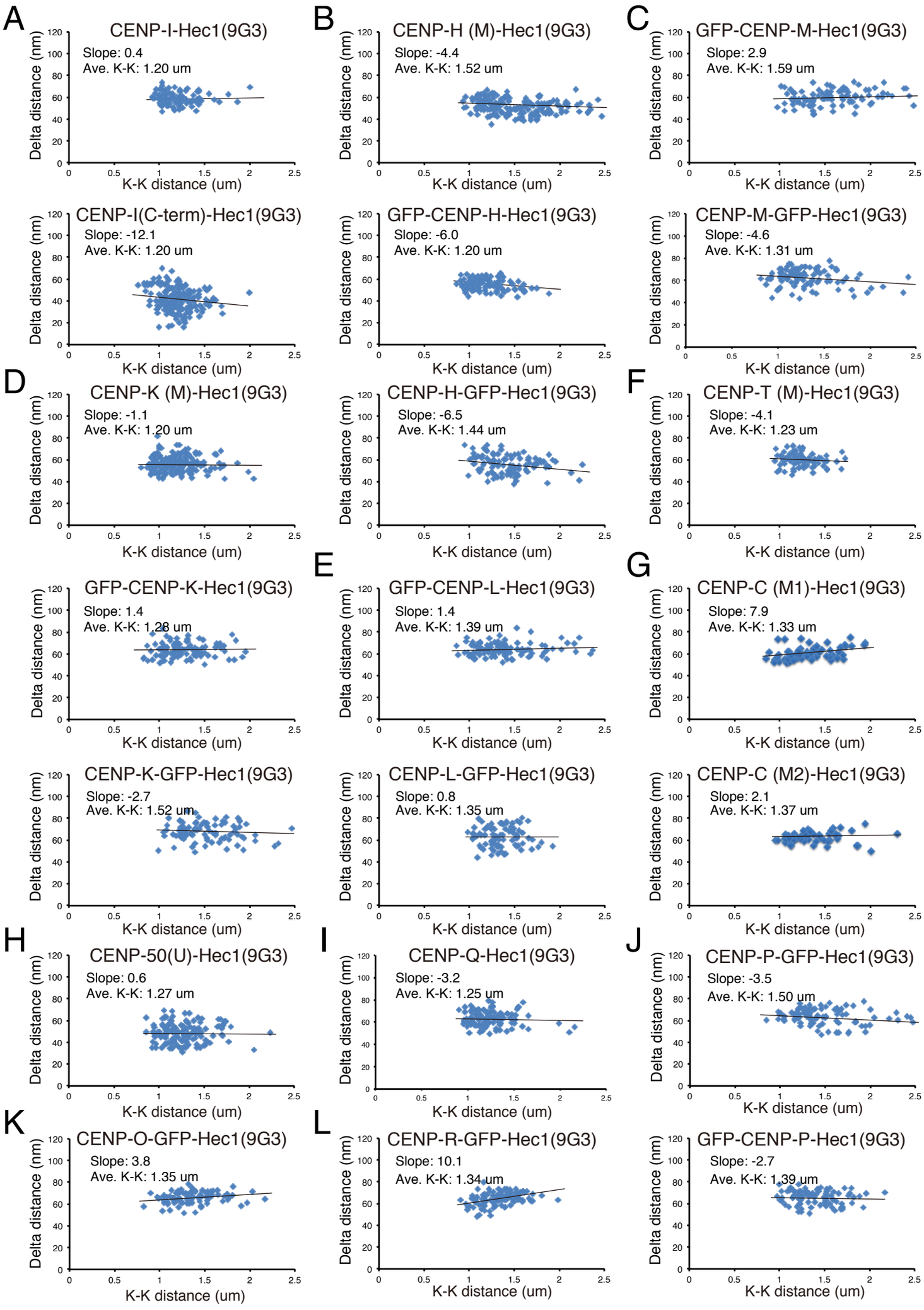
A
CENP-I-Hec1(9G3)
Slope: 0.4
Ave. K-K: 1.20 um
B
CENP-H (M)-Hec1(9G3)
Slope: -4.4
Ave. K-K: 1.52 um
C
GFP-CENP-M-Hec1(9G3)
Slope: 2.9
Ave. K-K: 1.59 um
CENP-I(C-term)-Hec1(9G3)
Slope: -12.1
Ave. K-K: 1.20 um
GFP-CENP-H-Hec1(9G3)
Slope: -6.0
Ave. K-K: 1.20 um
CENP-M-GFP-Hec1(9G3)
Slope: -4.6
Ave. K-K: 1.31 um
D
CENP-K (M)-Hec1(9G3)
Slope: -1.1
Ave. K-K: 1.20 um
CENP-H-GFP-Hec1(9G3)
Slope: -6.5
Ave. K-K: 1.44 um
F
CENP-T (M)-Hec1(9G3)
Slope: -4.1
Ave. K-K: 1.23 um
E
GFP-CENP-K-Hec1(9G3)
Slope: 1.4
Ave. K-K: 1.28 um
GFP-CENP-L-Hec1(9G3)
Slope: 1.4
Ave. K-K: 1.39 um
G
CENP-C (M1)-Hec1(9G3)
Slope: 7.9
Ave. K-K: 1.33 um
CENP-K-GFP-Hec1(9G3)
Slope: -2.7
Ave. K-K: 1.52 um
CENP-L-GFP-Hec1(9G3)
Slope: 0.8
Ave. K-K: 1.35 um
CENP-C (M2)-Hec1(9G3)
Slope: 2.1
Ave. K-K: 1.37 um
H
CENP-50(U)-Hec1(9G3)
Slope: 0.6
Ave. K-K: 1.27 um
I
CENP-Q-Hec1(9G3)
Slope: -3.2
Ave. K-K: 1.25 um
J
CENP-P-GFP-Hec1(9G3)
Slope: -3.5
Ave. K-K: 1.50 um
K
CENP-O-GFP-Hec1(9G3)
Slope: 3.8
Ave. K-K: 1.35 um
L
CENP-R-GFP-Hec1(9G3)
Slope: 10.1
Ave. K-K: 1.34 um
GFP-CENP-P-Hec1(9G3)
Slope: -2.7
Ave. K-K: 1.39 um
Delta distance (nm)
K-K distance (um)

Figure S1

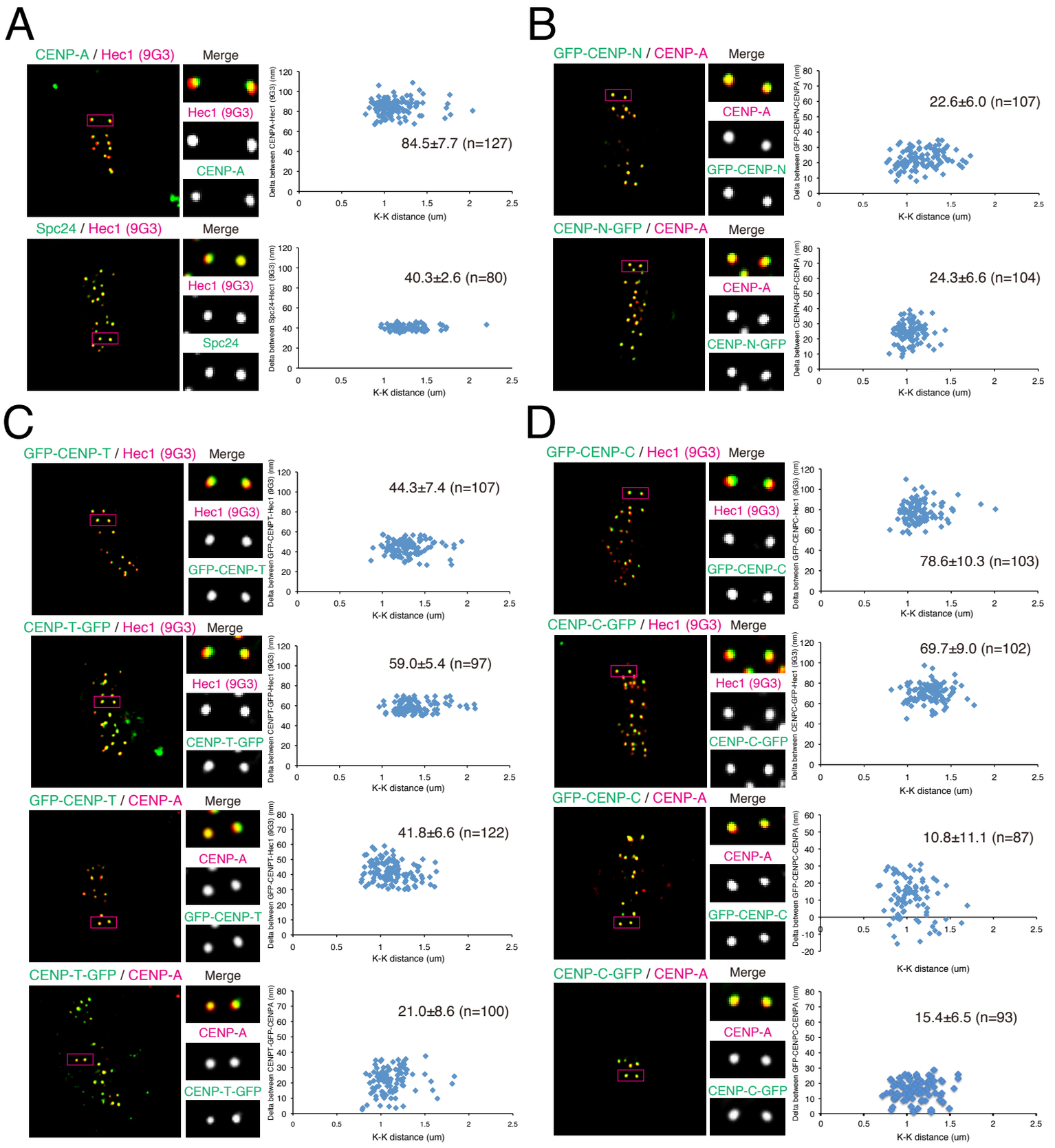
A
CENP-A / Hec1 (9G3)
Merge
Hec1 (9G3)
CENP-A
Delta between CENPA-Hec1 (9G3) (nm)
84.5±7.7 (n=127)
K-K distance (um)
Spc24 / Hec1 (9G3)
Merge
Hec1 (9G3)
Spc24
Delta between Spc24-Hec1 (9G3) (nm)
40.3±2.6 (n=80)
K-K distance (um)
B
GFP-CENP-N / CENP-A
Merge
CENP-A
GFP-CENP-N
Delta between GFP-CENPN-CENPA (nm)
22.6±6.0 (n=107)
K-K distance (um)
CENP-N-GFP / CENP-A
Merge
CENP-A
CENP-N-GFP
Delta between CENPN-GFP-CENPA (nm)
24.3±6.6 (n=104)
K-K distance (um)
C
GFP-CENP-T / Hec1 (9G3)
Merge
Hec1 (9G3)
GFP-CENP-T
Delta between GFP-CENPT-Hec1 (9G3) (nm)
44.3±7.4 (n=107)
K-K distance (um)
CENP-T-GFP / Hec1 (9G3)
Merge
Hec1 (9G3)
CENP-T-GFP
Delta between CENPT-GFP-Hec1 (9G3) (nm)
59.0±5.4 (n=97)
K-K distance (um)
GFP-CENP-T / CENP-A
Merge
CENP-A
GFP-CENP-T
Delta between GFP-CENPT-Hec1 (9G3) (nm)
41.8±6.6 (n=122)
K-K distance (um)
CENP-T-GFP / CENP-A
Merge
CENP-A
CENP-T-GFP
Delta between CENPT-GFP-CENPA (nm)
21.0±8.6 (n=100)
K-K distance (um)
D
GFP-CENP-C / Hec1 (9G3)
Merge
Hec1 (9G3)
GFP-CENP-C
Delta between GFP-CENPC-Hec1 (9G3) (nm)
78.6±10.3 (n=103)
K-K distance (um)
CENP-C-GFP / Hec1 (9G3)
Merge
Hec1 (9G3)
CENP-C-GFP
Delta between CENPC-GFP-Hec1 (9G3) (nm)
69.7±9.0 (n=102)
K-K distance (um)
GFP-CENP-C / CENP-A
Merge
CENP-A
GFP-CENP-C
Delta between GFP-CENPC-CENPA (nm)
10.8±11.1 (n=87)
K-K distance (um)
CENP-C-GFP / CENP-A
Merge
CENP-A
CENP-C-GFP
Delta between GFP-CENPC-CENPA (nm)
15.4±6.5 (n=93)
K-K distance (um)

Figure S2

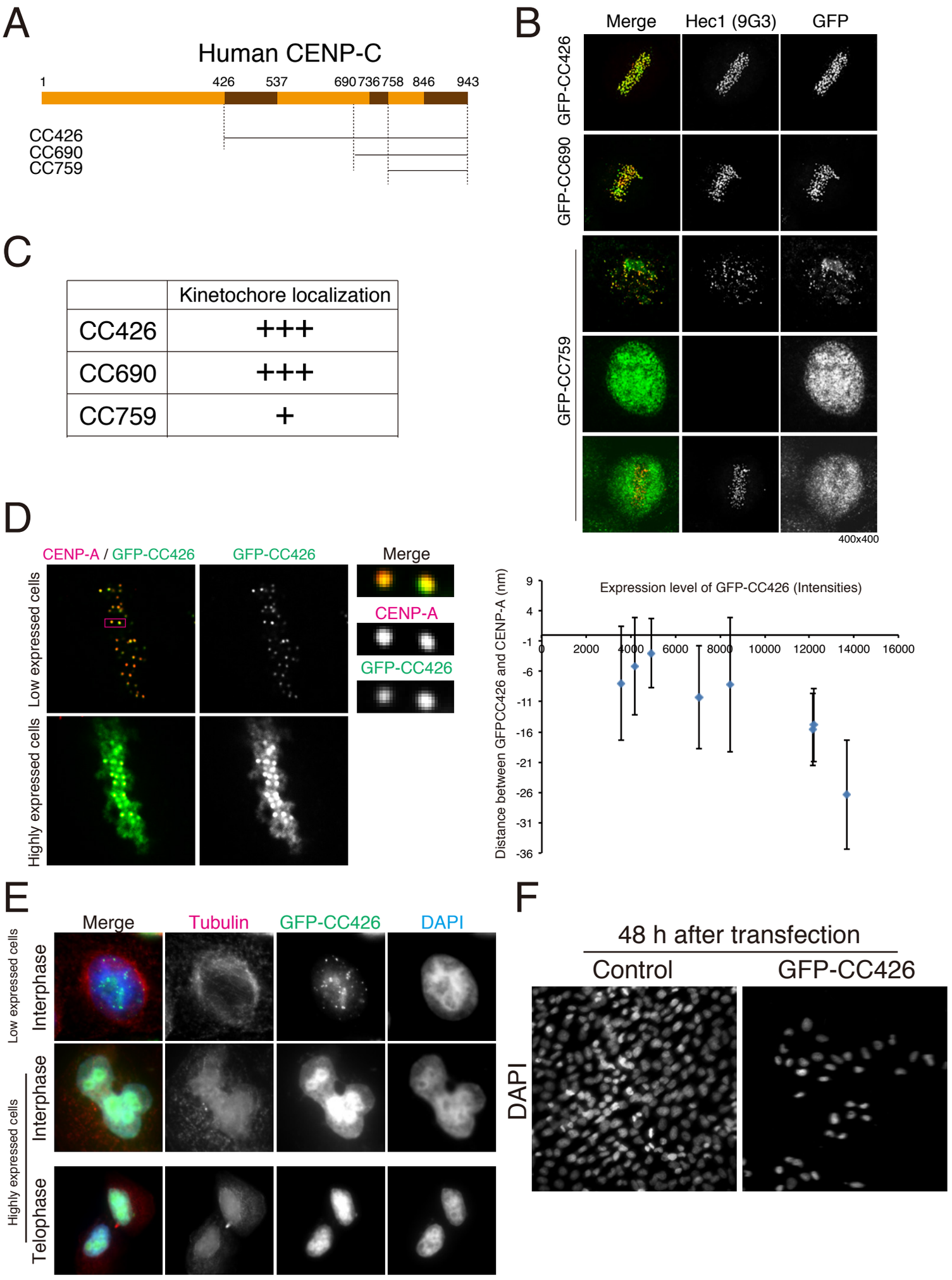
A
Human CENP-C
1
426
537
690
736
758
846
943
CC426
CC690
CC759
B
Merge
Hec1 (9G3)
GFP
GFP-CC426
GFP-CC690
GFP-CC759
400x400
C
Kinetochore localization
CC426
+++
CC690
+++
CC759
+
D
CENP-A / GFP-CC426
GFP-CC426
Merge
CENP-A
GFP-CC426
Low expressed cells
Highly expressed cells
Expression level of GFP-CC426 (Intensities)
Distance between GFPCC426 and CENP-A (nm)
9
4
-1
-6
-11
-16
-21
-26
-31
-36
0
2000
4000
6000
8000
10000
12000
14000
16000
E
Merge
Tubulin
GFP-CC426
DAPI
Low expressed cells
Interphase
Highly expressed cells
Interphase
Telophase
F
48 h after transfection
Control
GFP-CC426
DAPI

Figure S3

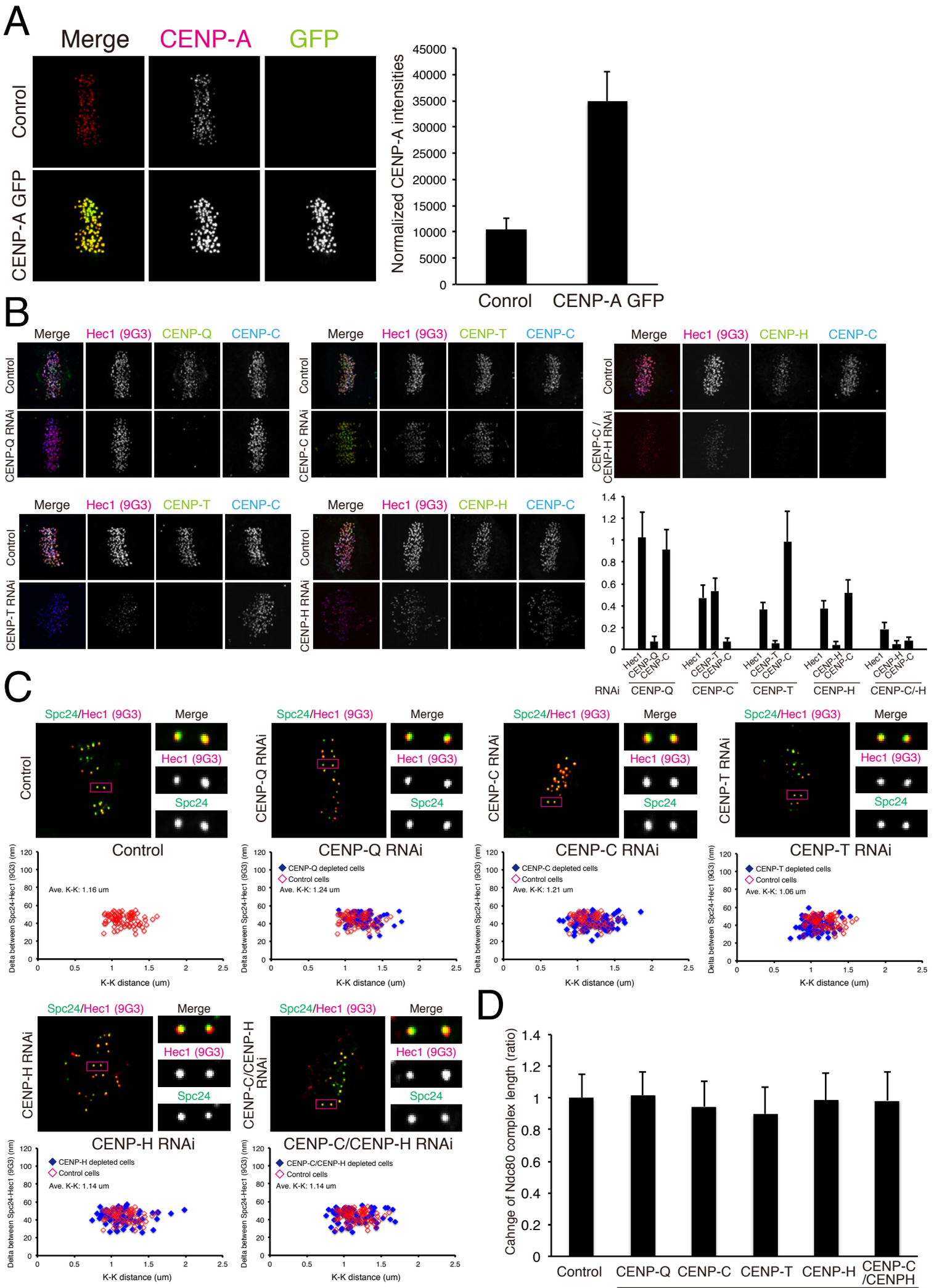
A
Merge
CENP-A
GFP
Conrol
CENP-A GFP
Normalized CENP-A intensities
45000
40000
35000
30000
25000
20000
15000
10000
5000
0
Conrol
CENP-A GFP
B
Merge
Hec1 (9G3)
CENP-Q
CENP-C
Control
CENP-Q RNAi
Merge
Hec1 (9G3)
CENP-T
CENP-C
Control
CENP-C RNAi
Merge
Hec1 (9G3)
CENP-H
CENP-C
Control
CENP-C / CENP-H RNAi
Merge
Hec1 (9G3)
CENP-T
CENP-C
Control
CENP-T RNAi
Merge
Hec1 (9G3)
CENP-H
CENP-C
Control
CENP-H RNAi
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Hec1
CENP-Q
CENP-C
Hec1
CENP-T
CENP-C
Hec1
CENP-T
CENP-C
Hec1
CENP-H
CENP-C
Hec1
CENP-H
CENP-C
RNAi
CENP-Q
CENP-C
CENP-T
CENP-H
CENP-C/-H
C
Spc24/Hec1 (9G3)
Merge
Hec1 (9G3)
Spc24
Control
CENP-Q RNAi
CENP-C RNAi
CENP-T RNAi
Control
Ave. K-K: 1.16 um
CENP-Q RNAi
CENP-Q depleted cells
Control cells
Ave. K-K: 1.24 um
CENP-C RNAi
CENP-C depleted cells
Control cells
Ave. K-K: 1.21 um
CENP-T RNAi
CENP-T depleted cells
Control cells
Ave. K-K: 1.06 um
Delta between Spc24-Hec1 (9G3) (nm)
K-K distance (um)
CENP-H RNAi
CENP-C/CENP-H RNAi
CENP-H RNAi
CENP-H depleted cells
Control cells
Ave. K-K: 1.14 um
CENP-C/CENP-H RNAi
CENP-C/CENP-H depleted cells
Control cells
Ave. K-K: 1.14 um
D
Cahnge of Ndc80 complex length (ratio)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Control
CENP-Q
CENP-C
CENP-T
CENP-H
CENP-C /CENPH
RNAi

Figure S4

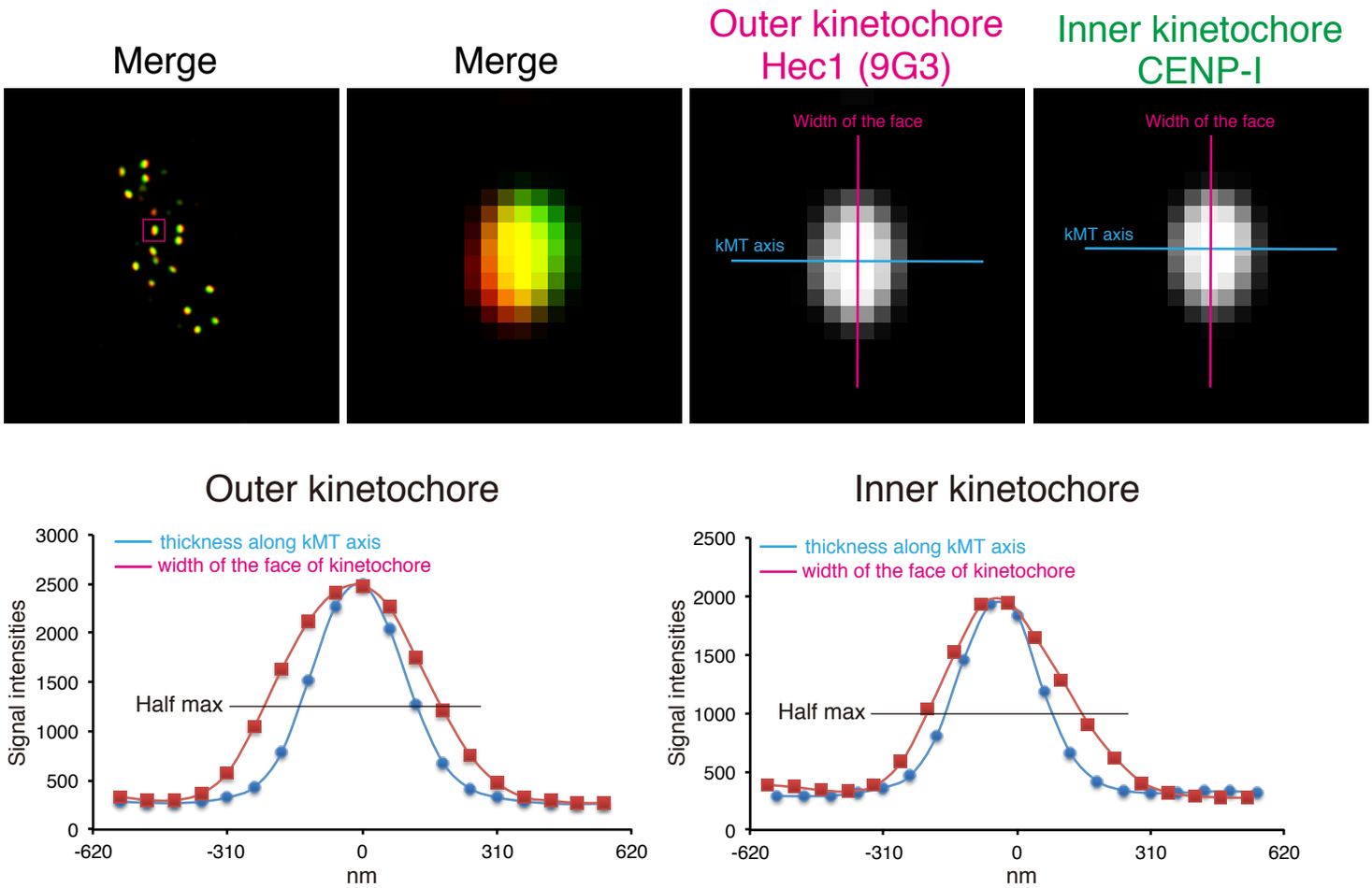
Merge
Merge
Outer kinetochore
Hec1 (9G3)
Inner kinetochore
CENP-I
Width of the face
kMT axis
Width of the face
kMT axis
Outer kinetochore
thickness along kMT axis
width of the face of kinetochore
Signal intensities
Half max
3000
2500
2000
1500
1000
500
0
-620
-310
0
310
620
nm
Inner kinetochore
thickness along kMT axis
width of the face of kinetochore
Signal intensities
Half max
2500
2000
1500
1000
500
0
-620
-310
0
310
620
nm

Figure S5

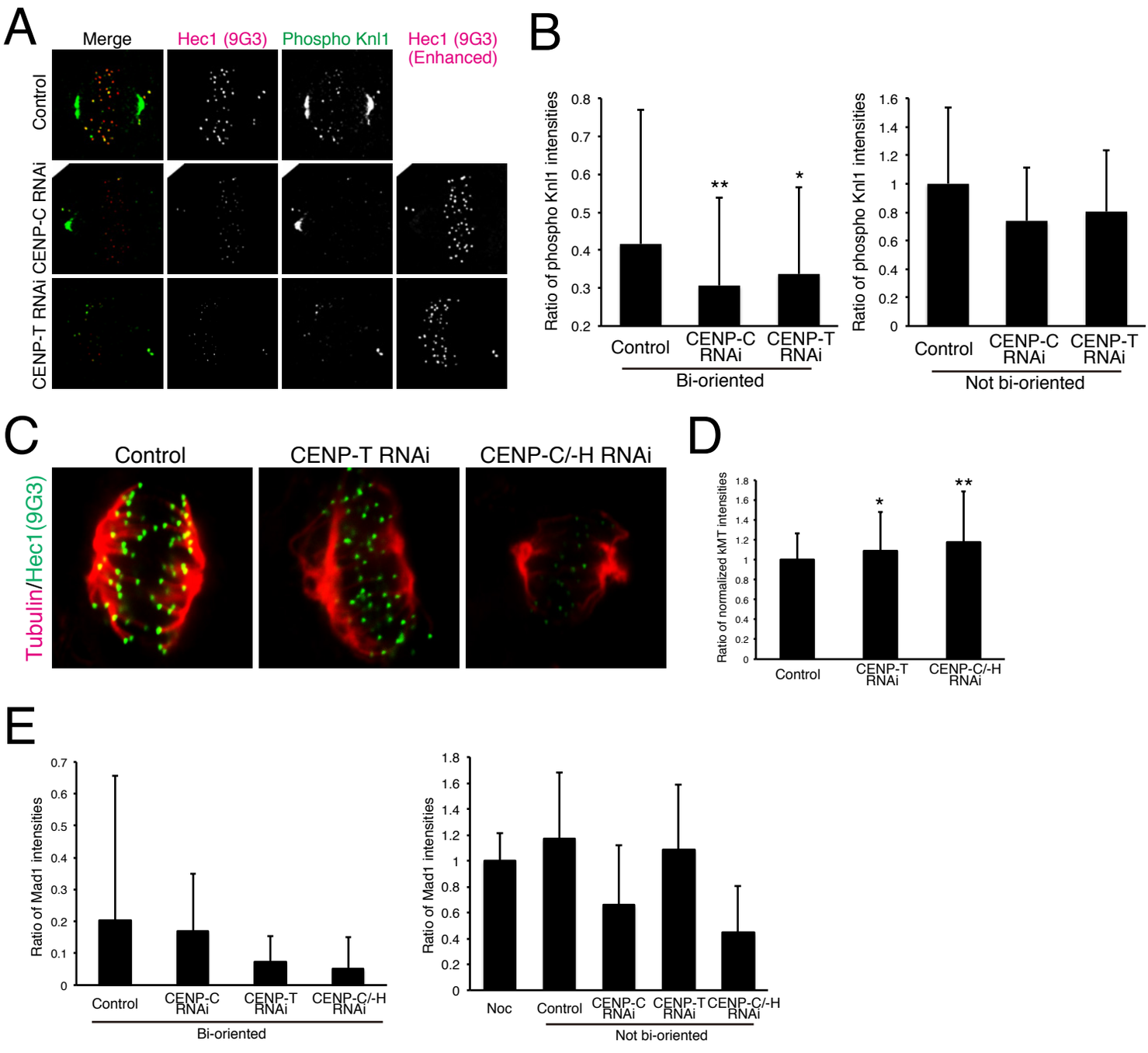
A
Merge
Hec1 (9G3)
Phospho Knl1
Hec1 (9G3) (Enhanced)
Control
CENP-C RNAi
CENP-T RNAi
B
Ratio of phospho Knl1 intensities
Control
CENP-C RNAi
CENP-T RNAi
Bi-oriented
Not bi-oriented
C
Control
CENP-T RNAi
CENP-C/-H RNAi
Tubulin/Hec1(9G3)
D
Ratio of normalized kMT intensities
E
Ratio of Mad1 intensities
Noc
Bi-oriented
Not bi-oriented

Figure S6