CENP-A Ubiquitylation Is Inherited through Dimerization between Cell Divisions

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Supplemental Experimental Procedures

Immunoblotting

The method of immunoblotting has been described in detail previously (Niikura et al., 2007; Niikura and Kitagawa, 2003; Niikura et al., 2015; Niikura et al., 2010; Niikura et al., 2006). Alternatively, the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE), the Odyssey CLx Infrared imaging System (LI-COR Biosciences, Lincoln, NE), and/or Molecular Imager Versadoc MP4000 System (Bio-Rad, Hercules, CA) were used for detection in the coimmunoblotting analysis.

In Figures 1B ("control"), 1C ("control"), 4B, S4B, and S4C, cells were cotransfected with CA-UTR #2 siRNAs (Table S2) to deplete endogenous CENP-A partially, but this partial depletion did not disrupt endogenous CENP-C localization at centromeres (ca. 80% of endogenous CENP-A remained at 48 h after transfection; data not shown).

To detect endogenous and exogenous CENP-A proteins in CENP-$A^{-/F}$ RPE1 cells, the experiment was performed in accordance with the time-course scheme shown in Figure S2A. Cells were seeded in a 6-well cell culture cluster (approximately 3.6×10^5 cells/well) and grown for 24 h before retro-Cre infection. Four days after retro-Cre infection, cells were infected with additional retroviruses harboring the indicated vector constructs (Table S3) and collected 6 days after retro-Cre infection.

Cells were suspended in denaturing buffer A2 (20 mM Tris-HCl pH 7.4, 50 mM NaCl, 0.5% Nonidet P-40, 0.5% deoxycholate, 0.5% SDS, 1 mM EDTA, and complete EDTA-free protease inhibitor cocktail [Roche]) (Wang et al., 2006) or buffer used in the immunoprecipitation assay (see the next section). The cell suspension was sonicated, frozen in liquid nitrogen, and thawed (freeze-thaw process). Before electrophoresis, cell lysates were mixed with SDS sample buffer (Lamb et al., 1995). The intensity of band signals was quantified by Quantity One 1-D Analysis Software Version 4.6.9 (Bio-Rad, Hercules, CA) or Image Studio Version 2.0-4.0 Software (LI-COR Biosciences, Lincoln, NE).

Immunoprecipitation Assay

The immunoprecipitation assay was performed as previously described (Niikura et al., 2007; Niikura and Kitagawa, 2003; Niikura et al., 2015; Niikura et al., 2010; Niikura et al., 2006) with the following minor modifications.

To study the interaction between N-terminal Flag-tagged CENP-A and N-terminal HA-tagged CENP-A (Figures 3A and S3H) and the interaction between N-terminal Flag-tagged CENP-A and endogenous HJURP or DAXX (Figures 6C and 6D), HeLa Tet-Off cells were cultured without tetracycline/doxycycline and transfected with the indicated plasmids (see Cell Culture and Transfection in Experimental Procedures). Forty-eight hours after transfection, cells were collected, dissolved in buffer C (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1.0% Nonidet P-40, 50 μM MG132, and complete EDTA-free protease inhibitor cocktail

[Roche]), and lysed by sonication and the freeze-thaw process. Proteins were immunoprecipitated with anti-Flag M2 affinity gel (SIGMA-ALDRICH). Immunoprecipitates were washed 4 times with buffer C, and proteins were eluted with SDS sample buffer (Lamb et al., 1995) and subjected to Western blot analysis with the indicated antibodies.

Immunofluorescence

Indirect immunofluorescent staining was performed as described previously (Niikura et al., 2007; Niikura and Kitagawa, 2003; Niikura et al., 2015; Niikura et al., 2010; Niikura et al., 2006) with the following minor modifications. HeLa or HeLa Tet-Off cells were grown for 48 h on coverslip slides after transfection with siRNA(s) (Table S2) and/or overexpression plasmid vector(s) (Table S3). Approximately 5.4×10^5 cells per well were seeded on cover glasses (22 mm × 22 mm) put in a 6-well polystyrene plate, and cells were grown for 18 h before transfection.

To detect endogenous CENP-A proteins in HeLa or HeLa Tet-Off cells, the cells were cultured without tetracycline/doxycycline and fixed 48 h after transfection with the indicated siRNA(s) (Table S2) plus vector construct(s) (Table S3). The cells were immunostained in accordance with the previously described method (Niikura et al., 2006) and the indicated antibodies (Table S1).

To detect exogenous N-terminal or C-terminal Flag-tagged CENP-A proteins in HeLa Tet-Off cells, the cells were cultured without

tetracycline/doxycycline and fixed 48 h after transfection with the indicated pTRM4-CENP-A construct(s) (Table S3). Cells were also cotransfected with CA-UTR #2 siRNAs (Table S2) to deplete endogenous CENP-A partially, but this partial depletion did not disrupt endogenous CENP-C localization at centromeres (ca. 80% of endogenous CENP-A remained at 48 h after transfection; data not shown). For the analysis of prophase and prometaphase cells only, Taxol (10 nM) was added 24 h after transfection, and cells were fixed 48 h after transfection.

To detect C-terminal Flag-tagged CENP-A proteins in HeLa Tet-Off cells, the asynchronous cells or cells treated with 10 nM Taxol for 24 h cells were fixed in 75% acetone at –20°C for 10 min. Cells were dried and treated with blocking buffer (0.5% skim milk and 0.5% BSA in PBS) at room temperature for 5 min. Cells were then incubated with a specific primary antibodies (Table S1) at 37°C for 1 h. After cells were washed with the blocking buffer, they were incubated with the Alexa fluor dye-conjugated secondary antibodies (Invitrogen; Table S1). Slides were washed twice with 0.1% skim milk and 0.1% BSA in PBS and then incubated in PBS containing 0.1 μg/ml DAPI.

To detect N-terminal Flag-tagged CENP-A proteins in HeLa Tet-Off cells, the asynchronous or Taxol-treated (10 nM for 24 h) cells were fixed in methanol at –20°C for 6 min. Cells were blocked with 4% goat serum in TBS at room temperature for 10 min. Cells were then incubated with a specific primary antibodies (Table S1) at 37°C for 1 h. After cells were washed with the blocking

buffer, they were incubated with the Alexa Fluor dye–conjugated secondary antibodies (Invitrogen; Table S1). Slides were washed twice with the blocking buffer and then incubated in TBS containing 0.1 μg/ml DAPI.

To detect endogenous and exogenous CENP-A proteins in CENP-$A^{-/F}$ RPE1 cells, the experiment was performed in accordance with the time-course scheme shown in Figure S2A. Cells were seeded in a 6-well cell culture cluster (approximately 3.6×10^5 cells/well) and grown for 24 h before retro-Cre infection. Alternatively, cells were re-seeded into an 8-well CC^2 treated glass slide (Lab-Tek™ II CC2™ Chamber Slide System; Nunc) 4 days after retro-Cre infection. Four days after retro-Cre infection, cells were infected with additional retroviruses harboring the indicated vector constructs. For mitotic cell analysis, Taxol (10 nM) or TN16 (0.5 μM) was added 24 h or 2.5 h before cell fixation, respectively. Cells were collected 6 days after retro-Cre infection and fixed and immunostained by using the previously described method (Niikura et al., 2006) with the indicated antibodies (Table S1). To detect N-terminal Flag-tagged CENP-A proteins in CENP-$A^{-/F}$ RPE1 cells, cells were fixed and stained as described for N-terminal Flag-tagged CENP-A proteins in HeLa Tet-Off cells (see the preceding text).

Image acquisition and processing, including deconvolution and signal quantitation, were performed by Openlab version 5.5.2 Scientific Imaging Software (Improvision, Lexington, MA) or Velocity version 6.3 3D Image Analysis Software (Improvision, Lexington, MA).

Chromosome Spreading

Samples were prepared as described (Kitajima et al., 2006) with the following minor modifications. In Figures 5, 6A, S1F, S3G, and S6, cells were also cotransfected with CA-UTR #2 siRNAs (Table S2) to deplete endogenous CENP-A partially, but this partial depletion did not disrupt endogenous CENP-C localization at centromeres (approximately 80% of endogenous CENP-A remained at 48 h after transfection; data not shown). For CENP-I, HEC1, HJURP, and DAXX costaining, HeLa Tet-Off cells were cultured for 46 h after transfection and treated with nocodazole (0.5 µg/ml) for 2 h. For SKA1 costaining, HeLa Tet-Off cells were treated with the double-thymidine block as described previously (Niikura et al., 2015), and cells were collected at approximately 10.5 h (during the mitotic phase) after release.

Cells were collected by trypsinization, gently resuspended in 0.2% (w/v) KCl and 0.2% (w/v) trisodium citrate hypotonic buffer, and incubated at room temperature (20–25°C) for 10 min. These hypotonically treated cells were sedimented onto slides by cytocentrifugation (Cytospin 4; ThermoShandon) at 600 rpm (41 *g*) for 3-5 min. The area around the spreads was marked with PAP-PEN (The Building Site, Inc.) and washed with 0.1% Triton X-100 in PBS at room temperature for at least 2 min before fixation. For the 3-color or 4-color staining of chromosomes, HeLa Tet-Off cells were fixed with 4% paraformaldehyde in PBS at 4°C for 15-30 min and treated with 0.5% Triton X-100 in KB2 (10 mM Tris-HCl pH 7.5, 150 mM NaCl, and 0.5% BSA) at room temperature for 30 min (or 8 min

only for SKA1 costaining). Cells were blocked with KB2 and incubated with a specific primary antibodies (Table S1) in KB2 at 37°C for 1 h. After cells were washed with KB2, they were incubated with the Alexa fluor dye-conjugated secondary antibodies (Invitrogen; Table S1) or other fluorescent secondary antibodies (Jackson Immuno Research; Table S1) at 37°C for 30 -60 min. After cells were washed with KB2, they were incubated with tertiary antibody at 37°C for 45-60 min as described in the following text. Slides were washed twice with KB2 and then incubated in KB2 containing 0.1 μg/ml DAPI (SIGMA-ALDRICH, St. Louis, MO).

For CENP-I, HEC1, HJURP, and DAXX costaining, Alexa Fluor 488–conjugated anti-Flag antibody (Cell signaling, Billerica, MA) was used as tertiary antibody after unbound secondary antibodies were washed away. For HJURP and DAXX costaining, another option for the tertiary antibody was anti-Flag antibody (SIGMA-ALDRICH, F7425; Table S1) labeled by the Zenon® Alexa Fluor® 488 Rabbit IgG Labeling Kit (Life Technologies, Grand Island, NY); for this option, cells were refixed with 4% paraformaldehyde in PBS at room temperature for 15 min after staining with tertiary antibody.

For SKA1 costaining, anti-SKA1 antibody (abcam; Table S1) was labeled by using the Zenon Alexa Fluor 488 Rabbit IgG Labeling Kit (Life Technologies) and applied after washing with KB2 blocking buffer as described in the preceding text. Cells were refixed with 4% paraformaldehyde in PBS at room temperature for 15 min after staining with tertiary antibody. Another option for the tertiary

antibody in SKA1 costaining was Alexa Fluor 488–conjugated anti-Flag antibody (Cell Signaling, Billerica, MA) ; for this option, cells were fixed with form fix (3.7% formaldehyde and 0.2% Triton X-100 in PEM buffer: 100 mM PIPES, 10 mM EGTA, and 1 mM $MgCl_2$ pH to 6.9 by KOH) (Foley et al., 2011) at room temperature for 10 min and pre-extracted with 0.5% Triton X-100 in PEM (100 mM PIPES, 10 mM EGTA, 1 mM MgCl2 pH to 6.9 by KOH) (Foley et al., 2011) at room temperature for 40 sec. Cells were blocked with PEM containing 2% donkey serum (PEM2) and incubated with a specific primary antibodies (Table S1) in the blocking buffer at 37°C for 1 h. After cells were washed with KB2, they were incubated with the Alexa Fluor dye-conjugated secondary antibodies (Invitrogen; Table S1) or other fluorescent secondary antibodies (Jackson Immuno Research; Table S1) at 37°C for 30-60 min. After cells were washed with PEM2, they were incubated with tertiary antibody at 37°C for 45-60 min. Slides were washed twice with PEM2 and then incubated in PEM containing 0.1 μg/ml DAPI (SIGMA-ALDRICH, St. Louis, MO).

More than 100 cells (Figures 5B, 5D, and 5G) or 50 cells (Figures 6B) were analyzed to obtain the average number of putative neocentromeres per cell, and more than 100 cells were analyzed to obtain the average number of “paired” putative neocentromeres with SKA1–positive sister chromatids per cell (Figure 5H).

Baculovirus and Human Recombinant Protein Expression Using Sf9 Insect

Cells

Recombinant human 6×His-tagged proteins (CENP-A WT, CENP-A H115A/L128A, CUL4A, RBX1, COPS8) and GST-tagged proteins (CENP-A K124R-Ub [K48R] and GST control protein) were expressed in Sf9 insect cells by using the Bac-to-Bac Baculovirus Expression System (Invitrogen) and Baculovirus Expression System with Gateway Technology (Invitrogen). Competent DH10Bac *E. coli* cells (Invitrogen) were transformed with Gateway pDEST10 or pDEST20 vector constructs (Invitrogen; Tables S3 and S4) to induce transposition. Recombinant bacmid DNA was selected, Sf9 insect cells were transfected with these bacmid DNA, and recombinant baculovirus particles were amplified (Table S4). Sf9 insect cells were infected with P1, P2, or higher−passage baculovirus, and cells were lysed in buffer D1 (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5% NP-40, and complete EDTA-free protease inhibitor cocktail [Roche]) or proper lysis buffer for each assay.

CENP-A In Vitro Ubiquitylation Assay

The CENP-A in vitro ubiquitylation assay was performed as described previously (Niikura et al., 2015) with the following minor modifications. The indicated 6xHis-tagged proteins (CENP-A WT and H115A/L128A, CUL4A, RBX1, and COPS8) and GST-tagged proteins (CENP-A K124R-Ub [K48R] and GST control protein) were expressed in Sf9 insect cells (Table S4; see Baculovirus and Human Recombinant Protein Expression Using Sf9 Insect Cells), purified, and eluted

(see Protein Purification). Plasmid pGEX 6P (modified)-GST-HJURP-6xHis (pDF263) was generously given by Dr. Daniel R. Foltz (Department of Biochemistry and Molecular Genetics, University of Virginia Medical School; Table S3). Recombinant human GST-HJURP- 6xHis was expressed and purified (see Protein Purification). Bacterially expressed 6xHis-tagged CENP-A protein was purified as described previously with a minor modification (Tanaka et al., 2004) (see Protein Purification).

Purified proteins were combined in a 12.5 μl reaction mixture that contained 50 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 2 mM ATP, 2 mM DTT, 0.2 μM ubiquitin aldehyde, 1 mM MG132, 2 μM LLnL, 100 ng E1 (UBE1), 100 ng E2 (UbcH5c), and 10 μg ubiquitin. Alternatively, these purified proteins were eluted with 12.5 μl of native elution buffer (see User Manual of Ni-NTA [nickel-nitrilotriacetic acid] Purification System, Invitrogen, 25-0490) and combined in12.5 μl of reaction mixture that contained 100 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 4 mM ATP, 4 mM DTT, 0.4 μM ubiquitin aldehyde, 2 mM MG132, 4 μM LLnL, 100 ng E1 (UBE1), 100 ng E2 (UbcH5c), and 10 μg ubiquitin.

If ubiquitylated CENP-A was depleted in Sf9 lysates (Figures 2B and S3B), in vitro pull-down assays were performed with Agarose-TUBE 2 (LifeSensors) in buffer D2 (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5% NP-40, 10 mM NaF, 1 mM PMSF, and complete EDTA-free protease inhibitor cocktail [Roche]). Subsequently the supernatant was used in the in vitro ubiquitylation. The purified proteins were eluted with 20 μl of buffer E (100 mM NaH_2PO_4, 1.0 M

NaCl, 500 mM imidazole, 20 mM reduced L-glutathione pH 8.0) and combined in 20 μl of reaction mixture that contained 100 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 4 mM ATP, 4 mM DTT, 0.4 μM ubiquitin aldehyde, 2 mM MG132, 4 μM LLnL, 133 ng E1 (UBE1), 133 ng E2 (UbcH5c), and 13.3 μg ubiquitin.

Protein Purification

Recombinant human 6×His-tagged CENP-A WT, CENP-A H115A/L128A, CUL4A, RBX1, and COPS8 proteins were expressed in Sf9 insect cells (Table S4; see Baculovirus and Human Recombinant Protein Expression Using Sf9 Insect Cells) and purified according to the manufacturer's instructions (see User Manual of Ni-NTA Purification System, Invitrogen, 25-0496). Recombinant human GST-CENP-A K 124R-Ub (K48R) and GST control proteins were expressed in Sf9 insect cells (Table S4; see Baculovirus and Human Recombinant Protein Expression Using Sf9 Insect Cells) and purified according to the manufacturer's instructions (see Instructions of Glutathione Sepharose 4 Fast Flow, GE Healthcare, 71-5016-97 AH). Alternatively, 6×His-tagged and GST-tagged protein purification was performed with buffer B1 (50 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 2 mM DTT, 50 μM MG132, and complete EDTA-free protease inhibitor cocktail [Roche]), buffer B2 (25 mM Tris-HCl pH 7.5, 4 mM $MgCl_2$, 0.1 mM DTT, and complete EDTA-free protease inhibitor cocktail [Roche]), or buffer D2 (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5% NP-40, 10 mM NaF, 1 mM PMSF, and complete EDTA-free protease inhibitor cocktail [Roche]). These proteins were

eluted with buffer E (100 mM NaH_2PO_4, 1.0 M NaCl, 500 mM imidazole, 20 mM reduced L-glutathione pH 8.0) (see CENP-A In Vitro Ubiquitylation Assay). The quality of purification was verified by use of SYPRO Ruby Protein Gel Stain (Molecular Probes) with SDS-polyacrylamide gels (Figure S3A).

Plasmid pGEX 6P (modified)-GST-HJURP-6xHis (pDF263) was generously given by Dr. Daniel R. Foltz (Department of Biochemistry and Molecular Genetics, University of Virginia Medical School; Table S3). Recombinant human GST-HJURP- 6xHis was expressed and purified as described previously (Foltz et al., 2009; Niikura et al., 2015), and the quality of purification was confirmed by SimplyBlue^{TM} SafeStain (Invitrogen) as described previously (Niikura et al., 2015).

Plasmid pHCE-6xHis-CENP-A was generously provided by Dr. Hitoshi Kurumizaka (Laboratory of Structural Biology, Graduate School of Advanced Science and Engineering, Waseda University, Japan; Table S3). Bacterially expressed 6xHis-tagged CENP-A protein was purified as described previously with a minor modification (Tanaka et al., 2004). Briefly, DH5α cells were transfected with pHCE-6xHis-CENP-A and 5 colonies of transformants were used to inoculate 1 L LB containing 50 μg/mL ampicillin and incubated at 37°C for 24 h. Cells were collected and lysed in 50 mL of buffer A (50 mM Tris-HCl pH 8.0, 500 mM NaCl, 1 mM PMSF, and 5% glycerol) by sonication. After centrifugation at 27,210 × *g* at 4°C for 20 min, the pellet containing insoluble 6xHis-CENP-A protein was dissolved in buffer A containing 7 M guanidine hydrochloride. 6xHis-

CENP-A was bound to Ni-NTA agarose resin (QIAGEN), and eluted with buffer A containing 6 M urea and 200 mM imidazole. The eluted proteins were bound to SP Sepharose Fast Flow (GE Healthcare), then eluted with buffer D (20 mM sodium acetate pH 5.2, 2 M NaCl, 5 mM β-mercaptoethanol, 1 mM EDTA, 6 M Urea). The eluted protein was dialyzed against PBS buffer containing 10% glycerol. The quality of purification was verified by staining SDS-polyacrylamide gels with SimplyBlue^{TM} SafeStain (Invitrogen) (Figure S3C).

LacO/LacI Ectopic Centromeric Chromatin Assembly System

Plasmids pcDNA3.1-HA-LacI, pcDNA3.1-HA-LacI-CENP-A WT, and U2OS-LacO cells were generously provided by Dr. Ben E. Black (Department of Biochemistry and Biophysics, Perelman School of Medicine, University of Pennsylvania; Table S3). Approximately 5.4×10^5 U2OS-LacO cells per well were seeded on cover glasses (22 mm × 22 mm) put in a 6-well polystyrene plate, and cells were grown for 18 h before transfection. Cells were transfected with the indicated construct(s) shown in Figure S7 by using linear polyethylenimine (PEI) (Reed et al., 2006) (see Cell Culture and Transfection in Experimental Procedures) and grown for 48 h on coverslip slides. For the analysis of HEC1 and SKA1, Taxol (10 nM) was added 24 h after transfection, and cells were fixed 48 h after transfection as described in the following text.

Indirect immunofluorescent staining was performed as described previously (Logsdon et al., 2015) with the following minor modifications. For

HEC1, SKA1, HJURP, or DAXX staining, cells were pre-extracted with 0.5% Triton X-100 in PBS for 3 min, fixed with 4% formaldehyde in PBS for 10 min, and then washed with 0.1% Tween in PBS for 5 min. Cells were blocked with 2% FBS, 2% BSA, and 0.1% Tween in PBS, and incubated with a specific primary antibodies (anti-DAXX antibody [abcam] was used for DAXX co-staining; Table S1) in the blocking buffer at room temperature for 1 h. After cells were washed with the blocking buffer, they were incubated with the Alexa Fluor dye-conjugated secondary antibodies (Invitrogen; Table S1) at 1:300 dilution at 37°C for 30 min. Slides were washed twice with the blocking buffer and then incubated in PBS containing 0.1 μg/ml DAPI (SIGMA-ALDRICH, St. Louis, MO). For DAXX staining, cells were fixed in 4% formaldehyde in PBS for 10 min, extracted with 0.5% Triton X-100 in PBS for 5 min, and then washed in 0.1% Tween in PBS for 5 min 3 times prior to blocking and antibody incubation. Cells were blocked with 20% FBS, 3% BSA, and 0.1% Tween in PBS, and incubated with a specific primary antibodies (anti-DAXX antibody [Cell Signaling] was used for DAXX costaining; Table S1) in the blocking buffer at room temperature for 1 h. After cells were washed with the blocking buffer, they were incubated with the Alexa Fluor dye-conjugated secondary antibodies (Invitrogen; Table S1) at a 1:300 dilution ratio at 37°C for 30 min. Slides were washed twice with the blocking buffer and then incubated in PBS containing 0.1 μg/ml DAPI (SIGMA-ALDRICH, St. Louis, MO). Image acquisition and quantitation were performed as described previously (Niikura et al., 2015).

Table S1. Antibodies Used in This Study, Related to Experimental Procedures

Antibody	Antibody type	Source	Catalog/Database number
Anti-CENP-A	Mouse monoclonal	Stressgen / Enzo Life Sciences	KAM-CC006
Anti-CENP-A	Rabbit polyclonal	Upstate	07-574
Anti-CENP-B	Mouse monoclonal	Novus Biologicals	H00001059-B01P
Anti-CENP-B	Rabbit polyclonal	abcam	ab25734
Anti-CENP-C	Rabbit sera	This study	-
Anti-CENP-I	Rabbit sera	This study	-
Anti-HEC1	Mouse monoclonal	Novus Biologicals / GeneTex	NB100-338 / GTX70268
Anti-HEC1	Rabbit polyclonal	GeneTex	GTX110735
Anti-SKA1	Rabbit polyclonal	abcam	ab46826
Anti-Centromere (CREST sera)	Human sera	Fitzgerald Industries Intl.	90C-CS1058
Anti-HJURP	Rabbit polyclonal	Proteintech Group Inc.	15283-1-AP
Anti-DAXX	Rabbit polyclonal	abcam	ab105173
Anti-DAXX	Rabbit polyclonal	Cell Signaling	4533
Anti-GST	Rabbit polyclonal	abcam	ab9085
Anti-Flag	Mouse monoclonal	SIGMA-ALDRICH	F3165
Anti-Flag	Rabbit polyclonal	SIGMA-ALDRICH	F7425
Anti-Flag (Alexa Fluor 488 conjugated)	Rabbit polyclonal	Cell Signaling	5407
Anti-HA	Mouse monoclonal	Roche	1666606
Anti-HA	Rabbit polyclonal	Santa Cruz	SC-805
Anti-5XHis	Mouse monoclonal	QIAGEN	34660
Anti-GAPDH	Mouse monoclonal	Chemicon	MAB374
Anti-GAPDH	Rabbit polyclonal	abcam	ab37168
Anti–β-tubulin	Mouse monoclonal	MP Biomedicals	63781
Anti–β-tubulin	Rabbit polyclonal	abcam	ab18587

goat anti-mouse IgG-HRP	Affinity-purified secondary antibody	Santa Cruz	SC-2005
goat anti-rabbit IgG-HRP	Affinity-purified secondary antibody	Santa Cruz	SC-2004
goat anti-rabbit IgG-HRP	Affinity-purified secondary antibody	Cell Signaling	7074
goat anti-rabbit IgG-HRP	Affinity-purified secondary antibody	Jackson ImmunoResearch	111-035-144
IRDye 800CW Goat Anti-Mouse IgG	Affinity-purified secondary antibody	LI-COR Biosciences	926-32210
IRDye 680 Goat Anti-Rabbit IgG	Affinity-purified secondary antibody	LI-COR Biosciences	926-32221
Alexa Fluor 488 Goat Anti-Mouse IgG	Affinity-purified secondary antibody	Invitrogen	A11001
Alexa Fluor 594 Goat Anti-Mouse IgG	Affinity-purified secondary antibody	Invitrogen	A11005
Alexa Fluor 488 Goat Anti-Rabbit IgG	Affinity-purified secondary antibody	Invitrogen	A11008
Alexa Fluor 594 Goat Anti-Rabbit IgG	Affinity-purified secondary antibody	Invitrogen	A11012
Alexa Fluor 594 Goat Anti-Human IgG	Affinity-purified secondary antibody	Invitrogen	A11014
DyLight 647 Donkey Anti-Mouse IgG	Affinity-purified secondary antibody	Jackson Immuno Research Laboratories	715-605-150
Cy3-conjugated AffiniPure Donkey Anti-Rabbit IgG	Affinity-purified secondary antibody	Jackson Immuno Research Laboratories	711-165-152

Table S2. siRNA Sequences Used in This Study, Related to Experimental Procedures

siRNA target	Indication in present study	Target type	siRNA database number	Forward sequence(s)	Source/Reference	Catalog/ID number
Luciferase (GL3)	-	1 target	RKK9	CUUACGCUGAGUACUUCGAdTdT	Elbashir et al., (2001)	-
CENP-A	CA-UTR #1	siRNA pool (10 targets mixture)	RKK375 / 5' UTR t1	CGAGCGGCGCGGACUUCUGCCdTdT	this study (1)	
			RKK383 / 3' UTR t1	UCCUGCACCCAGUGUUUCUGUdGdT	this study (1)	
			RKK459 / 3' UTR t4	GGAUUCUGCGAUGCUGUCU	this study (1)	
			RKK461 / 3' UTR t5	GCGAUGCUGUCUGGACUUU	this study (1)	
			RKK463 / 3' UTR t6	UCAGAUAAAGAGACUCCAA	this study (1)	
			RKK465 / 3' UTR t7	GGCUGGGCAUUUCCAUCAU	this study (1)	
			RKK467 / 3' UTR t8	GCUGGGCAUUUCCAUCAUA	this study (1)	
			RKK469 / 3' UTR t9	CCAUUAGUGGCAGCAUCAU	this study (1)	
			RKK471 / 3' UTR t10	GCUUUGAUGUUCUGGUUAC	this study (1)	
			CRKK167 / RKK547 / 3' UTR t12	AAGAUGUAUCAUAACAGUUCA	this study (1)	SI00343182
	CA-UTR #2	siRNA pool (2 targets mixture)	RKK375 / 5' UTR t1	CGAGCGGCGCGGACUUCUGCCdTdT	this study (1)	
			RKK383 / 3' UTR t1	UCCUGCACCCAGUGUUUCUGUdGdT	this study (1)	
HJURP	-	siRNA pool (3 targets mixture)	CRKK264 / RKK503 / t1	CAAGUAUGGAGGUUCGAUAdTdT	AMBION	s30814
			CRKK265 / RKK505 / t2	GAAGGAAUCGUUACGAUGAdTdT	AMBION	s30815
			CRKK266 / RKK507 / t3	GUAUCUACUUGAUUAACCAdTdT	AMBION	s30816

(1) Synthesized by the Hartwell Center for Bioinformatics and Biotechnology, St. Jude Children's Research Hospital.

Table S3. Plasmid/Bacmid Vectors Used in This Study, Related to Experimental Procedures

B number	Relevant characteristic(s)	Source	Reference
B288	pTRM4	-	(Niikura et al., 2006) / This study
B2067	pTRM4-human CENP-A WT-Flag	-	(Niikura et al., 2015) / This study
B2512	pTRM4-Flag-human CENP-A WT	-	(Niikura et al., 2015) / This study
B2579	pTRM4-Flag-human CENP-A K124R	-	(Niikura et al., 2015) / This study
B2560	pTRM4-Flag-human CENP-A K124R-Ub (K48R)	-	(Niikura et al., 2015) / This study
B3022	pTRM4-Flag-human CENP-A H115A/L128A	-	This study
B3036	pTRM4-Flag-human CENP-A H115A/L128A-Ub (K48R)	-	This study
B2881	pTRM4-human CENP-A	-	This study
B2781	pTRM4-human CENP-A K124R-Ub (K48R)	-	This study
B2806	pCGN-HA-Ubiquitin	-	(Niikura et al., 2015) / This study
B3036	pTRM4-Flag-human CENP-A H115A/L128A-PD	-	This study
B3065	pTRM4-Flag-human CENP-A H115A/L128A-ND	-	This study

B3067	pTRM4-human CENP-A WT-PD	-	This study
B3069	pTRM4-human CENP-A WT-ND	-	This study
B3028	pBabe-puro	Dr. Amruta Ashtekar / Dr. Lawrence S. Kirschner	This study
B3027	pBabe-puro-Cre	Dr. Amruta Ashtekar / Dr. Lawrence S. Kirschner	This study
B3031	psPAX2	Dr. John Thompson / Dr. Gustavo W. Leone	This study
B3032	pMD2.G	Dr. John Thompson / Dr. Gustavo W. Leone	This study
B2993	pQCXIP-Flag-human CENP-A	-	This study
B3029	pQCXIP-Flag	-	This study
B2999	pQCXIP-HA-human CENP-A	-	This study
B3038	pQCXIP-HA-human CENP-A H115AL128A	-	This study
B3045	pQCXIP- human CENP-A	-	This study
B3033	pQCXIP-human CENP-A K124R-Ub (K48R)	-	This study
B3046	pQCXIP	-	This study
B1197	pCMV-VSG-G	-	This study
B1242	pDEST20-GST	-	(Niikura et al., 2015) / This study
B2927	pDEST20-GST-human CENP-A K124R-Ub (K48R)	-	This study
B2916	pDEST10-6xHis-human CENP-A WT	-	This study
B3025	pDEST10-6xHis-human CENP-A H115A/L128A	-	This study
B2498	pDEST10-6xHis-human CUL4A	-	(Niikura et al., 2015) / This study
B2508	pDEST10-6xHis-human RBX1/ROC1	-	(Niikura et al., 2015) / This study
B2438	pDEST10-6xHis-human COPS8/CSN8	-	(Niikura et al., 2015) / This study
B2985	pGEX 6P (modified)-GST-human HJURP-6xHis (pDF263)	Dr. Daniel R. Foltz	(Niikura et al., 2015)
B3080	pHCE-6xHis-human CENP-A	Dr. Hitoshi Kurumizaka	(Tanaka et al., 2004)
B3097	pcDNA3.1-HA-LacI	Dr. Ben E. Black	(Logsdon et al., 2015)
B3096	pcDNA3.1-HA-LacI-human CENP-A WT	Dr. Ben E. Black	(Logsdon et al., 2015)

B3104	pcDNA3.1-HA-LacI-human CENP-A K124R	-	This study
B3106	pcDNA3.1-HA-LacI-human CENP-A K124R-Ub (K48R)	-	This study

Table S4. Baculovirus Extracts Used in This Study, Related to Experimental Procedures

Baculovirus database number	Protein expressed	B number of recombinant donor plasmid (see Table S3)	Relevant characteristic(s) of bacmid	Reference
Bv48	GST	B1242	bMON14272-GST	(Niikura et al., 2015) / This study
Bv100	GST-human CENP-A K124R-Ub (K48R)	B2927	bMON14272-GST-human CENP-A K124R-Ub (K48R)	This study
Bv94	6xHis-human CENP-A WT	B2916	bMON14272-6xHis-human CENP-A WT	(Niikura et al., 2015) / This study
Bv121	6xHis-human CENP-A H115A/L128A	B3025	bMON14272-6xHis-human CENP-A H115A/L128A	This study
Bv73	6xHis-human CUL4A	B2498	bMON14272-6xHis-human CUL4A	(Niikura et al., 2015) / This study
Bv75	6xHis-human RBX1/ROC1	B2508	bMON14272-6xHis-human RBX1/ROC1	(Niikura et al., 2015) / This study
Bv67	6xHis-human COPS8/CSN8	B2438	bMON14272-6xHis-human COPS8/CSN8	(Niikura et al., 2015) / This study

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Supplemental Figure Legends

Figure S1. Supplemental data related to Figure 1.

(A) Western blot analysis of total lysates of HeLa Tet-Off cells transfected with CENP-A siRNAs targeting 5' UTR and 3' UTR sequences (Table S2, CA-UTR #1) or Luc siRNA. Cells were cultured without tetracycline/doxycycline. Endogenous CENP-A was detected with an anti-CENP-A antibody. GAPDH protein was used as a loading control. Percentage (%) of anti-CENP-A Western blot signals compared with the signal of the Luc siRNA sample is indicated (normalized to anti-GAPDH signals).

(B) Reduction to less than 10% of the endogenous CENP-A level by CENP-A UTR-targeting siRNAs (Table S2, CA-UTR #1) disrupted centromere assembly (Delocalization of CENP-C in response to CA-UTR #1 siRNA treatment). HeLa cells were transfected for 48 h with CA-UTR #1 siRNAs or Luc siRNA. Immunostaining results with DAPI (blue), CENP-C (green), and CENP-B (red) at prophase, metaphase, and interphase are shown. Scale bar, 10 μm.

(C) CENP-C signals at centromeres given in (B) were quantified. Signals were normalized with those in Luc siRNA-treated cells, and mean percentages (±SD) are shown. $^{****}P < 0.0001$ and $^{***}P < 0.001$ compared with Luc siRNA-treated cells (Student's *t* test).

(D) Representative images of other cell cycle stages (related to Figure 1D). HeLa Tet-Off cells were cultured, cotransfected with indicated constructs (Table S3)

plus siRNAs (Table S2, CA-UTR #1 or #2 [control]), and immunostained as described for Figure 1D (see Immunofluorescence in Supplemental Experimental Procedures). DAPI (blue), Flag (green), and endogenous CENP-B (red) as a centromere localization control were visualized. Asterisks indicate a non-transfected Flag-negative cell. Scale bar, 10 μm.

(E) Histograms summarizing the localization patterns shown D) (related to Figure 1E). More than 50 pro/prometaphase and 200 interphase cells were counted per experiment ($n \geq 3$ experiments), and the mean percentages (±SD) are shown. "Others (Non-centromere)" indicates mostly damaged cells, dead cells, or cells with nucleolar localization in interphase, presumably because of transfection or other treatments. $^{****}P < 0.0001$, $^{***}P < 0.001$, $^{**}P < 0.01$, and $^{*}P < 0.05$ compared with the sample [2] (Student's *t* test).

(F) Chromosome spreads were prepared from HeLa Tet-Off cells transfected and cultured as in Figures 1D, 1E, S1D, and S1E (see Chromosome Spreading in Supplemental Experimental Procedures). Forty-six hours after transfection, cells were arrested in the mitotic phase by treatment with nocodazole for 2 h, and their chromosomes were stained with antibodies against Flag (CENP-A) and CENP-B. DNA was costained with DAPI. Representative images including magnified paired sister chromatids are shown (insets). K124R-Ub (K48R): pTRM4-Flag-CENP-A K124R-Ub (K48R), WT: pTRM4-Flag-CENP-A WT transfectant (Table S3). Scale bar, 10 μm or 2 μm.

(G and H) Confirmation of overexpression of (G) untagged CENP-A K124R-Ub

(K48R) indicated in Figures 1A-1E, S1D, S1E, S1I, and S1J; and (H) untagged CENP-A WT indicated in Figures S1I and S1J. HeLa Tet-Off cells were cultured without tetracycline/doxycycline and harvested 48 h after transfection with (G) pTRM4-CENP-A K124R-Ub (K48R), (H) pTRM4-CENP-A WT, or pTRM4 vector. Overexpression was confirmed by Western blot analysis with the anti-CENP-A antibody. GAPDH protein was used as a loading control. Putative CENP-A dimer (##) and CENP-A monomer (#) are indicated. SDS-resistant CENP-A dimers have been reported previously (Shelby et al., 1997; Yoda et al., 2000). Ubiquitylated CENP-A bands did not appear on this membrane because the samples did not express HA-Ub, were not treated with MG132, and did not undergo immunoprecipitatation.

(I) Exogenous “untagged” CENP-A does not localize to centromeres when cells are depleted of pre-existing endogenous CENP-A. HeLa Tet-Off cells were cultured and cotransfected as in Figures 1D and S1D but with the indicated constructs and siRNAs (Tables S2 and S3; see also Figures 1B, 1C, S1G, and S1H). Cells were immunostained as in Figure S1B. DAPI (blue), endogenous and/or exogenous CENP-A (green), and endogenous CENP-B (red) as a centromere localization control were visualized. Cells with a “diffuse” type of localization as in Figures 1D and S1D were observed but not included in these images (see [J] below). Vec: pTRM4-vector, CENP-A WT-Flag: pTRM4-CENP-A WT-Flag, Untagged CENP-A WT-Flag: pTRM4-CENP-A WT, Untagged CENP-A

K124R-Ub (K48R): pTRM4-CENP-A K124R-Ub (K48R) transfectant (Table S3). Scale bar, 10 μm.

(J) CENP-A signals at centromeres given in (I) were quantified. Signals were normalized with those of Luc siRNA plus vector-transfected cells (i.e., sample [1]), and the mean percentages (± SD) are shown. Cells with a “diffuse” type of localization as in Figures 1D and S1D were observed in (I) but not included in this quantitation of total CENP-A signals that used anti-CENP-A antibody; the purpose was to fairly compare with sample [1] and to not underestimate the remaining centromere signals (%) of the sample [4]. ****$P < 0.0001$ compared with the sample [1] (Student’s *t* test).

Figure S2. Supplemental data related to Figures 1.

(A) Top, Western blot analysis of total lysates of CENP-A$^{-/F}$ RPE1 cells infected with a retrovirus harboring Flag-CENP-A 4 days after retro-Cre infection (Table S3; see Immunoblotting in Supplemental Experimental Procedures). Cells were cultured and infected with retroviruses harboring the indicated vector constructs, and total lysates were analyzed at the indicated time points. Endogenous CENP-A and exogenous CENP-A-Flag protein levels were detected with anti-CENP-A antibody and anti-Flag antibody, respectively. GAPDH protein was used as a loading control. Percentage (%) of anti-CENP-A Western blot signals set to 100% with the day-0 point sample (for endogenous CENP-A) after retro-Cre infection is indicated (normalized with anti-GAPDH signals). Bottom, histogram shows

quantified endogenous and exogenous CENP-A. Percentages (%) of anti-CENP-A and anti-Flag Western blot signals set to 100% with day 0 (for endogenous CENP-A) and day 6 (for exogenous Flag-CENP-A) samples after retro-Cre infection are shown, respectively (normalized with anti-GAPDH signals). The day on which Flag-CENP-A overexpression began is indicated with an arrow (i.e., 4 days after retro-Cre infection).

(B) In vivo ubiquitylation assay using total lysates of CENP-$A^{-/F}$ RPE1 cells transfected/infected with pCGN-HA-Ubiquitin and retroviruses harboring the indicated vector constructs 2 days after infection of Flag-CENP-A-expressing retrovirus (see In Vivo CENP-A Ubiquitylation Assay in Experimental Procedures). Proteins in 5% of the total cell lysates (input) and immunoprecipitates (IP) were detected by Western blot analysis using the indicated antibodies. K124R-Ub (K48R): pQCXIP-CENP-A K124R-Ub (K48R), Vec: pQCXIP-vector transfectant (Table S3).

(C) Confirmation of overexpression constructs by Western blot analysis of CENP-$A^{-/F}$ RPE1 cell total lysates. Cells were infected for 48 h with a retrovirus harboring untagged CENP-A WT or untagged CENP-A K124R-Ub (K48R) or a mock virus of vector transfectant (Table S3). Retro-Cre infection was not performed and endogenous CENP-A bands remained on this blot. Ubiquitylated CENP-A bands did not appear on this membrane because the samples did not coexpress HA-Ub, were not treated with MG132, and did not undergo immunoprecipitation. Untagged WT: pQCXIP-CENP-A WT, untagged K124R-Ub

(K48R): pQCXIP-CENP-A K124R-Ub (K48R), Vector: pQCXIP- vector transfectant (Table S3). GAPDH protein was used as a loading control.

(D) Immunostaining of CENP-A$^{-/F}$ RPE1 cells infected with retroviruses harboring the indicated vector constructs (Table S3) (see Cell Culture and Transfection in Experimental Procedures and Immunofluorescence in Supplemental Experimental Procedures). DAPI (blue), Flag (green), and endogenous CENP-B (red) as a centromere localization control were visualized. Vec: pQCXIP-vector, K124R-Ub (K48R): pQCXIP-CENP-A K124R-Ub (K48R) transfectant (Table S3). Scale bar, 10 μm.

(E) Histograms summarizing the localization patterns shown in (D). More than 50 pro/prometaphase cells, 50 metaphase cells, and 200 interphase cells were counted per experiment ($n \geq 3$ experiments), and the mean percentages (±SD) are shown. "Others (Non-centromere)" indicates mostly cells that were damaged or died, presumably because of transfection or other treatments. $^{****}P < 0.0001$ compared with the sample in the center column (Student's *t* test) (see Immunofluorescence in Supplemental Experimental Procedures).

(F) Exogenous untagged CENP-A does not localize to centromeres when cells are depleted of pre-existing endogenous CENP-A in CENP-A$^{-/F}$ RPE1 cells. Cells were cultured, infected with retroviruses harboring the indicated vector constructs, and immunostained (see Cell Culture and Transfection in Experimental Procedures and Immunofluorescence in Supplemental Experimental Procedures). DAPI (blue), endogenous and/or exogenous CENP-A

(green), and endogenous CENP-B (red) as a centromere localization control were visualized by using the same method as described for Figure S1I. Vec-untagged: pQCXIP-vector, untagged CENP-A: pQCXIP-CENP-A, Vec-Flag: pQCXIP-Flag vector, Flag-CENP-A: pQCXIP-Flag-CENP-A transfectant (Table S3). Scale bar, 10 μm.

(G) CENP-A signals at centromeres in (F) were quantified. Signals were normalized with the sample [1], and the mean percentages (±SEM) calculated by the same method as Figure S1J are shown. $^{****}P < 0.0001$ compared with the sample [1] (Student's *t* test).

Figure S3. Supplemental data related to Figures 2 and 3.

(A) Purification of 6xHis-CENP-A WT, 6xHis-CENP-A H115A/L128A, 6xHis-CUL4A, 6xHis-RBX1, 6xHis-COPS8, GST-CENP-A K124R-Ub (K48R), and GST proteins from Sf9 lysates was verified by use of SYPRO Ruby Protein Gel Stain (Molecular Probes) staining. BSA was used as a quantity control.

(B) Adding HJURP after removal of ubiquitylated CENP-A from Sf9 lysates enhanced CENP-A ubiquitylation in vitro (see CENP-A In Vitro Ubiquitylation Assay in Supplemental Experimental Procedures). The molecular size of ubiquitylated 6xHis-CENP-A (6xHis-CENP-A-Ub) was larger than that of 6xHis-COPS8, unlike 6xHis-CENP-A purified from bacteria cells shown in (D), because bMON14272-6xHis-CENP-A had a longer linker sequence than pHCE-6xHis-CENP-A (Tables S3 and S4). Putative 6xHis-CENP-A-Ub is indicated by the

arrow. The histogram shows the quantified putative 6xHis-CENP-A-Ub band (the ratio of the ubiquitylated band signal to total CENP-A band signal which included nonubiquitylated CENP-A band signal, was normalized with the sample 2). Experiments were repeated ($n \geq 3$ experiments), and the mean percentages (±SD) are shown. ** $P < 0.01$ comparing samples 4 and 5 (Student's *t* test).
(C) Purification of 6xHis-CENP-A WT protein from bacteria was verified by staining with SimpleBlue™ SafeStain (Invitrogen). BSA was used as a quantity standard. Asterisk * indicates non specific bands.

(D) Adding HJURP to CENP-A purified from bacteria enhanced CENP-A ubiquitylation in vitro (see CENP-A In Vitro Ubiquitylation Assay in Supplemental Experimental Procedures). The molecular size of ubiquitylated 6xHis-CENP-A (6xHis-CENP-A-Ub) was smaller than that of 6xHis-COPS8, unlike 6xHis-CENP-A purified from Sf9 cells shown in (B), because pHCE-6xHis-CENP-A had a shorter linker sequence than bMON14272-6xHis-CENP-A (Tables S3 and S4). Putative 6xHis-CENP-A-Ub is indicated by the arrow. The histogram shows the quantified putative 6xHis-CENP-A-Ub band (the ratio of the ubiquitylated band signal to total CENP-A band signal which included the nonubiquitylated CENP-A band signal, was normalized with sample 3). Experiments were repeated ($n \geq 3$ experiments), and the mean percentages (±SD) are shown. ** $P < 0.01$ comparing the samples 3 and 4 (Student's *t* test).

(E) Representative images of the in vivo ubiquitylation assay with the combination of HJURP or Luc siRNA. The enhanced endogenous HJURP protein

level that resulted from CENP-A-Flag overexpression (input, middle lane) was reduced with HJURP siRNA treatment (input, right lane). β-tubulin protein was used as a loading control (input). The bottom of the histogram shows quantified ubiquitylated CENP-A bands (the ratio of Flag band signal was normalized to the sample in the left column). Experiments were repeated ($n \geq 3$ experiments), and the mean percentages (±SD) are shown. $^{****}P < 0.0001$ and $^{**}P < 0.01$ compared with the sample in the left column (Student's *t* test).

(F) The CENP-A H115A/L128A mutation reduces dimerization of CENP-A in total cell lysates. HeLa Tet-Off cell total lysates were subjected to Western blot analysis. Cells were cultured without tetracycline/doxycycline and harvested 48 h after transfection with each construct (Table S3). Putative dimers (##) and monomers (#) of each construct are indicated. Ubiquitylated CENP-A bands did not appear in this blot because the samples did not coexpress HA-Ub, were not treated with MG132, and did not undergo immunoprecipitatation. Vec: pTRM4 vector, WT: pTRM4-Flag-CENP-A WT, H115A/L128A: pTRM4-Flag-CENP-A H115A/L128A transfectant (Table S3). GAPDH protein was used as a loading control.

(G) Chromosome spreads were prepared from HeLa Tet-Off cells transfected and cultured as shown in Figures 3C and 3D (see Chromosome Spreading in Supplemental Experimental Procedures). Forty-six hours after transfection, cells were arrested in the mitotic phase by treatment with nocodazole for 2 h, and their chromosomes were stained with antibodies against Flag (CENP-A) and CENP-B.

DNA was costained with DAPI. Representative images, including those of magnified paired sister chromatids, are shown (insets). WT: pTRM4-Flag-CENP-A WT, H115A/L128A: pTRM4-Flag-CENP-A H115A/L128A transfectant (Table S3). Scale bar, 10 μm or 2 μm.

(H) CENP-A monoubiquitin fusion does not rescue the dimerization of CENP-A. The in vivo immunoprecipitation assay was performed using constructs shown in the upper table. HeLa Tet-Off cells were cultured and transfected with pQCXIP-HA-CENP-A (WT) plus pTRM4-Flag-CENP-A (WT, H115A/L128A, or K124R), pTRM4-Flag-CENP-A (WT, H115A/L128A, or K124R)-Ub (K48R), or pTRM4 vector (Table S3; see Immunoprecipitation Assay in Supplemental Experimental Procedures). Proteins in 3% of the total cell lysates (Input) and immunoprecipitates (IP) obtained by using ANTI-FLAG M2 Affinity Gel (SIGMA-ALDRICH) were detected by immunoblotting using the indicated antibodies.

Figure S4. Supplemental data related to Figure 4.

(A) Schematic figures of each construct used in this study. The N-terminal Flag tag on CENP-A (white “F” in black rectangle), the H115A/L128A mutation sites (red double bars), and dimerization domain (black “D” in the orange rectangle) are indicated. Two types of dimerization domains, ScPUT3 (PD) and DmNcd (ND), are also indicated. The status of centromere localization of each Flag-tagged CENP-A protein matches that of ubiquitylation (right two columns).

(B) Western blot analysis of HeLa Tet-Off cell total lysates. Cells were cultured without tetracycline/doxycycline and harvested 48 h after cotransfection with each construct indicated as in (A) (see also Table S3) plus CA-UTR #2 siRNA (the exception was sample [11], which had been transfected with the pTRM4 vector and Luc siRNA; see Table S2 and Immunoblotting in Supplemental Experimental Procedures). Putative dimers (##) and monomers (#) of each construct are indicated. Putative dimer bands are mixture of homodimers (i.e., homodimer of Flag-CENP-A WT, homodimer of Flag-CENP-A H115A/L128A-D, and/or homodimer of untagged CENP-A WT-D) and heterodimers (i.e., heterodimer of Flag-CENP-A H115A/L128A-D and untagged CENP-A WT-D) among expressed constructs in each sample. Using an anti-CENP-A antibody, Western blot analysis of samples [6] and [9] presumably showed bands of homodimers of untagged CENP-A WT-D; these bands were absent when the blot was analyzed with an anti-Flag antibody. Western blot analysis of samples [5] and [6] showed doublet bands representative of two monomers: the upper band was untagged WT-PD and the lower band was Flag-WT or Flag-H1125A/L128A. Unlike [5] and [6], analysis of samples [8] and [9] did not show doublet bands when anti-CENP-A antibody was used, because ND (24 amino acids) is shorter than PD (28 amino acids). Therefore, unlike untagged WT-PD, untagged WT-ND was inseparable from Flag-WT or Flag-H1125A/L128A in the present 15% SDS-PAGE gel. The amount of dimers formed through ND is presumably larger than that formed through PD; therefore, the intensity of anti-Flag signal and anti-

CENP-A signal of the dimer bands from samples [8] and [10] is slightly greater than that from samples [5] and [7]. Ubiquitylated CENP-A bands did not appear on this membrane because the samples did not coexpress HA-Ub, were not treated with MG132, did not undergo immunoprecipitatation.

(C) In vivo ubiquitylation assay (related to Figure 4B; see CENP-A In Vivo Ubiquitylation Assay in Experimental Procedures). HeLa Tet-Off cells were cotransfected with the indicated constructs as numbered in (A) and (B). Proteins in 5% of the total cell lysates (Input) and immunoprecipitates (IP) were detected by Western blot analysis using the indicated antibodies.

Figure S5. Supplemental data related to Figure 4.

(A) Representative images of cells during metaphase (related to Figure 4C and images of samples [1]-[4], [7], and [10] are the same as in Figure 4C; representative data of other cell-cycle stages are shown in Figures S5C and S5E). HeLa Tet-Off cells were cultured, cotransfected with indicated constructs and siRNAs (Tables S2 and S3), and immunostained as in Figure 4C (see Immunofluorescence in Supplemental Experimental Procedures). DAPI (blue), Flag (green), and endogenous CENP-B (red), which served as a centromere localization control, were visualized. Scale bar, 10 μm.

(B) Histograms summarizing the localization patterns shown in (A) (related to Figure 4D and data of samples [1]-[4], [7], and [10] are the same as in Figure 4D; representative data of other cell-cycle stages are shown in Figures S5D and

S5F). More than 50 metaphase cells were counted per experiment ($n \geq 3$ experiments), and the mean percentages (±SD) are shown. “Others (Non-centromere)” indicates mostly damaged cells or dead cells presumably because of transfection or other treatments. ****$P < 0.0001$ compared with the sample [1] (Student’s *t* test).

(C) Representative images of cells during prophase (related to Figure 4C). HeLa Tet-Off cells were cultured, cotransfected with indicated constructs and siRNAs (Tables S2 and S3), and immunostained as in Figure 4C (see Immunofluorescence in Supplemental Experimental Procedures). DAPI (blue), Flag (green), and endogenous CENP-B (red), which served as a centromere localization control, were visualized. Scale bar, 10 μm.

(D) Histograms summarizing the localization patterns shown in (C) (related to Figure 4D). More than 50 pro/prometaphase cells were counted per experiment ($n \geq 3$ experiments), and the mean percentages (±SD) are shown. “Others (Non-centromere)” indicates mostly damaged cells or dead cells presumably because of transfection or other treatments. ****$P < 0.0001$, ***$P < 0.001$, and *$P < 0.05$ compared with the sample [1] (Student’s *t* test).

(E) Representative images of cells during interphase (related to Figure 4C). HeLa Tet-Off cells were cultured, cotransfected with indicated constructs and siRNAs (Table S2 and S3), and immunostained as in Figure 4C (see Immunofluorescence in Supplemental Experimental Procedures). DAPI (blue), Flag (green), and endogenous CENP-B (red), which served as a centromere

localization control, were visualized. Scale bar, 10 μm.

(F) Histograms summarizing the localization patterns shown in (E) (related to Figure 4D). More than 200 interphase cells were counted per experiment ($n \geq 3$ experiments), and the mean percentages (±SD) are shown. "Others (Non-centromere)" indicates mostly damaged cells, dead cells, or cells with nucleolar localization in interphase, presumably because of transfection or other treatments. $^{****}P < 0.0001$ and $^{**}P < 0.01$ compared with the sample [1] (Student's *t* test).

Figure S6. Supplemental data related to Figures 4.

Chromosome spreads were prepared from HeLa Tet-Off cells transfected and cultured as described for Figures 4C, 4D, and S5 (see Chromosome Spreading in Supplemental Experimental Procedures). Forty-six hours after transfection, cells were arrested in the mitotic phase by treatment with nocodazole for 2 h, and their chromosomes were stained with antibodies against Flag (CENP-A) and CENP-B. DNA was costained with DAPI. Representative images, including images of magnified paired sister chromatids, are shown (insets). Sample numbers [1]-[10] correspond to those shown in Figures 4, S4, and S5. Scale bar, 10 μm or 2 μm.

Figure S7. CENP-A Ubiquitylation Contributes to Recruitment of CENP-A Chaperons and Outer Kinetochore Proteins to LacO arrays in the LacO/LacI

Ectopic Centromeric Chromatin Assembly System, Related to Figures 5 and 6.

(A) Confirmation of overexpression of the indicated HA-LacI constructs used in Figure S7. U2OS-LacO cells were cultured and harvested 48 h after transfection with the indicated pcDNA3.1-HA-LacI constructs. Overexpression was confirmed by Western blot analysis with the indicated antibodies. GAPDH protein was used as a loading control.

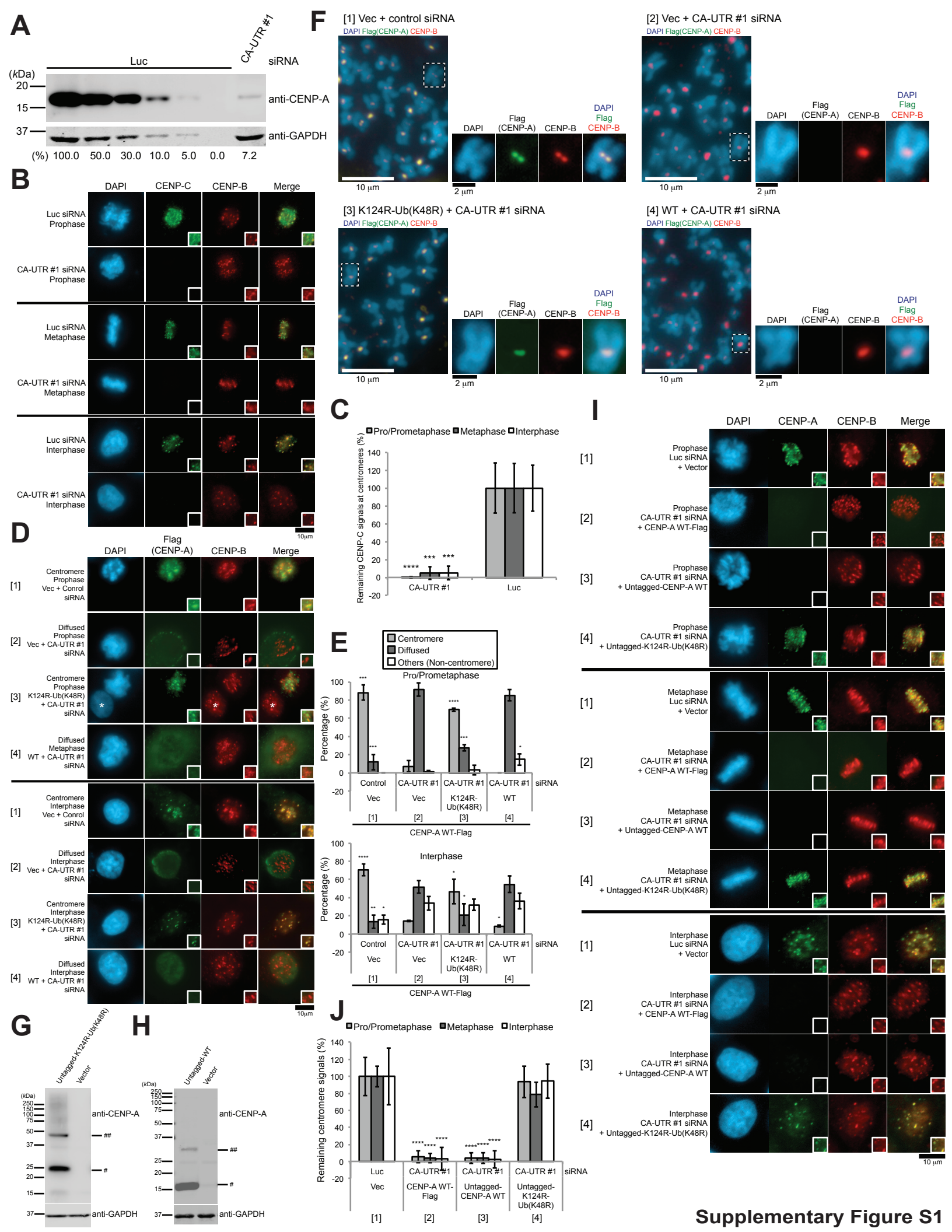
(B) K124R-Ub (K48R) mutant substantially restored the centromere localization of CENP-A, which was abolished by K124R mutant. The histogram summarizes the localization patterns shown in representative images of Figures S7C and S7E ('HA' columns). More than 50 pro/prometaphase cells, 50 metaphase cells, and 200 interphase cells were counted per experiment ($n \geq 3$ experiments), and the mean percentages (±SD) are shown. "Others (Non-centromere)" indicates mostly damaged cells, dead cells, or cells with nucleolar localization in interphase, presumably because of transfection or other treatments. $^{****}P < 0.0001$, $^{**}P < 0.01$, and $^{*}P < 0.05$ compared with WT (Student's *t* test).

(C) Ubiquitylation of LacI-fused CENP-A is important for efficient recruitment of HEC1 and SKA1 to LacO arrays. Representative immunofluorescent images of HEC1 and SKA1 recruitment to LacO arrays in prometaphase cells by the indicated HA-LacI-fused constructs are shown. DAPI (blue), HA (green; low and high brightness), and endogenous HEC1 or SKA1 (red) were visualized. Scale bar, 10 μm.

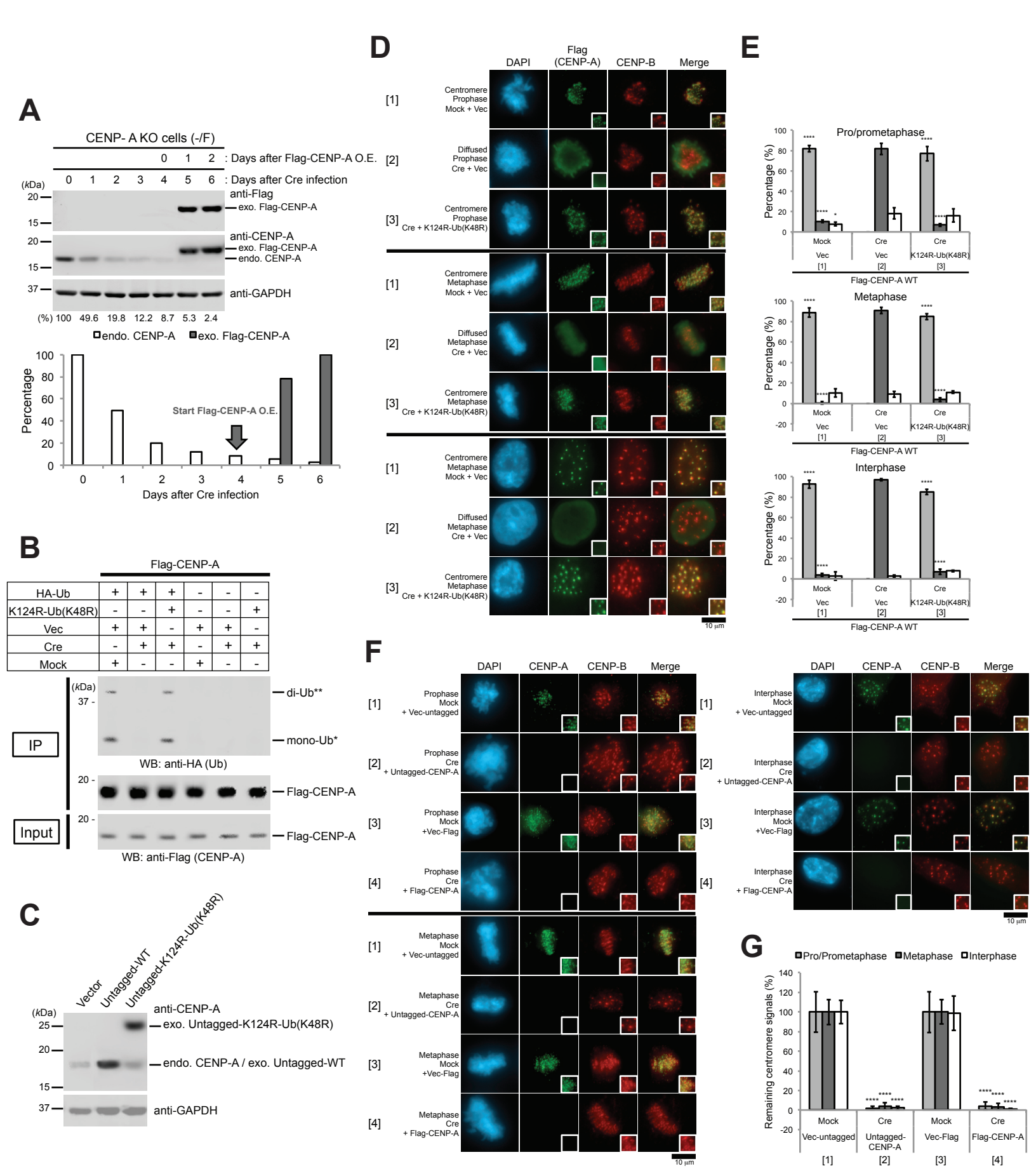
(D) The histogram shows quantified HEC1 and SKA1 fluorescent intensity at LacO arrays of the indicated HA-LacI constructs shown in (C) (ratio of the HEC1 or SKA1 intensity to HA intensity at LacO arrays was normalized with WT). More than 20 individual signals at LacO arrays in pro/prometaphase cells were quantified, and the mean percentages (±SEM) are shown. *** $P < 0.001$, ** $P < 0.01$, and * $P < 0.05$ comparing the indicated two data sets (Student's *t* test).

(E) Ubiquitylation of LacI-fused CENP-A is important for efficient recruitment of HUJRP and DAXX to the ectopic sites. Representative immunofluorescent images of HJURP and DAXX recruitment to LacO arrays in interphase cells by the indicated HA-LacI-fused constructs are shown. DAPI (blue), HA (green; low and high brightness), and endogenous HJURP or DAXX (red) were visualized. Scale bar, 10 μm.

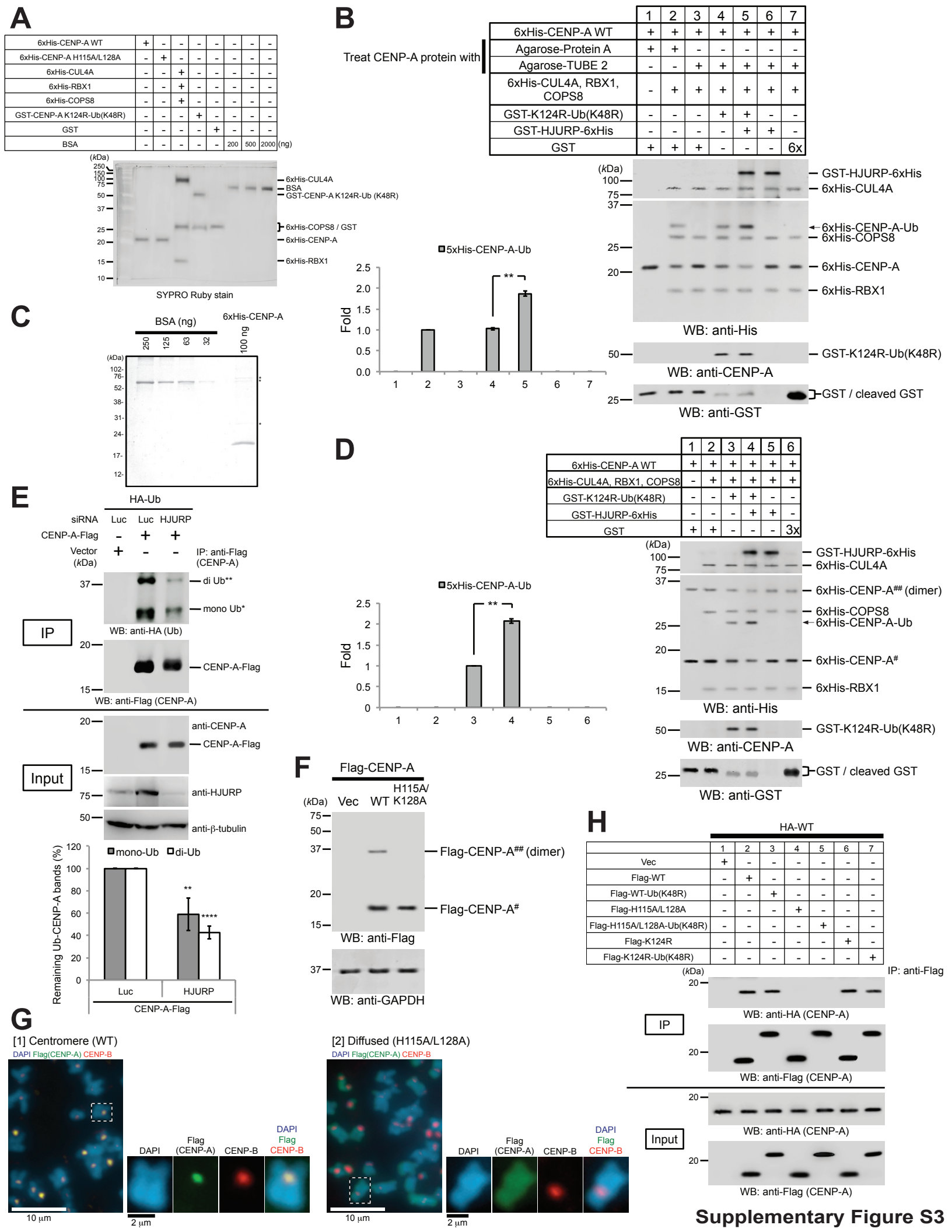
(F) The histogram shows quantified HEC1 and SKA1 fluorescent intensity at LacO arrays of the indicated HA-LacI constructs shown in (E) (ratio of the HJURP or DAXX intensity to HA intensity at LacO arrays was normalized with WT). More than 20 individual signals at LacO arrays in interphase cells were quantified, and the mean percentages (±SEM) are shown. **** $P < 0.0001$, ** $P < 0.01$, and * $P < 0.05$ comparing the indicated two data sets (Student's *t* test).



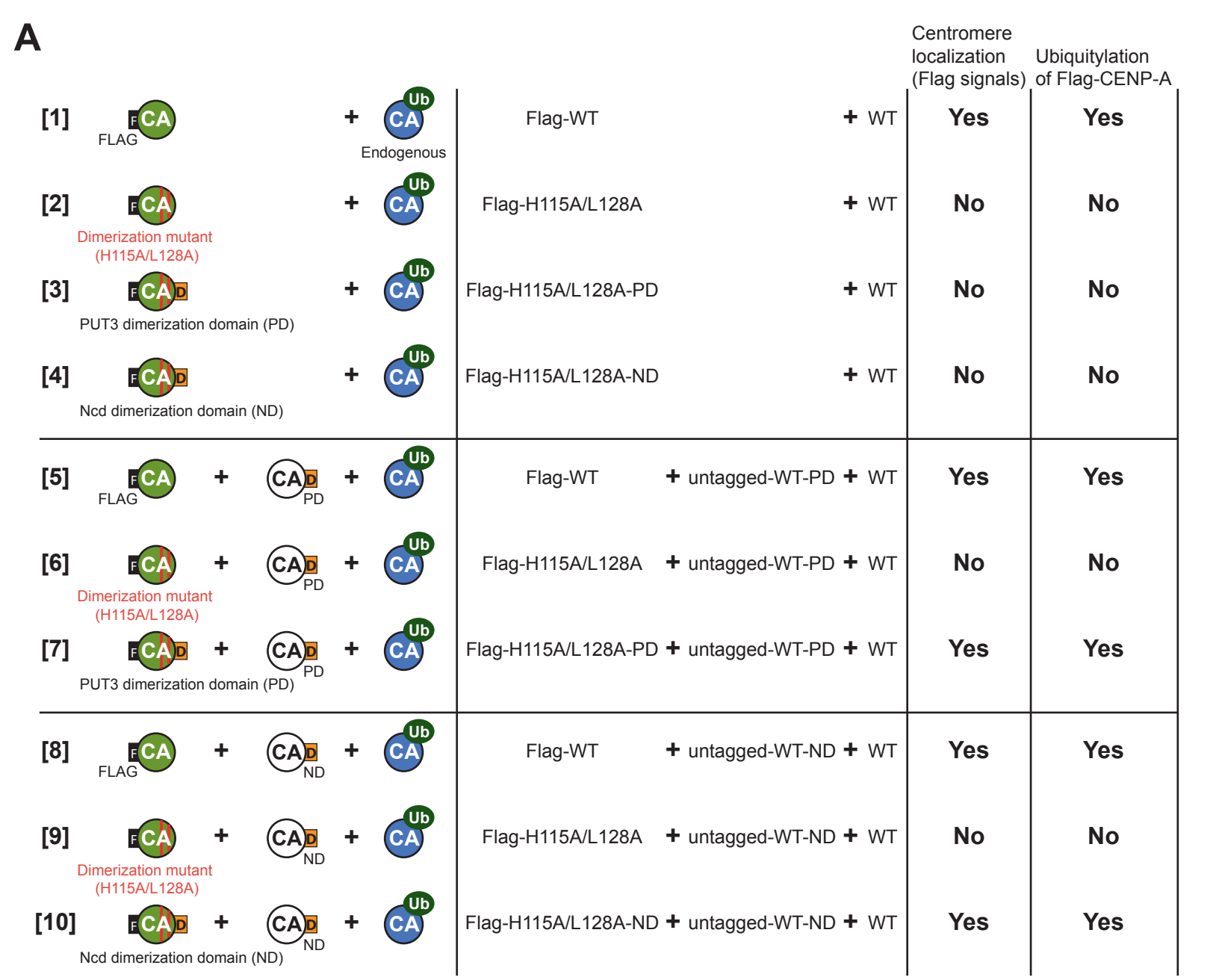
Supplementary Figure S1



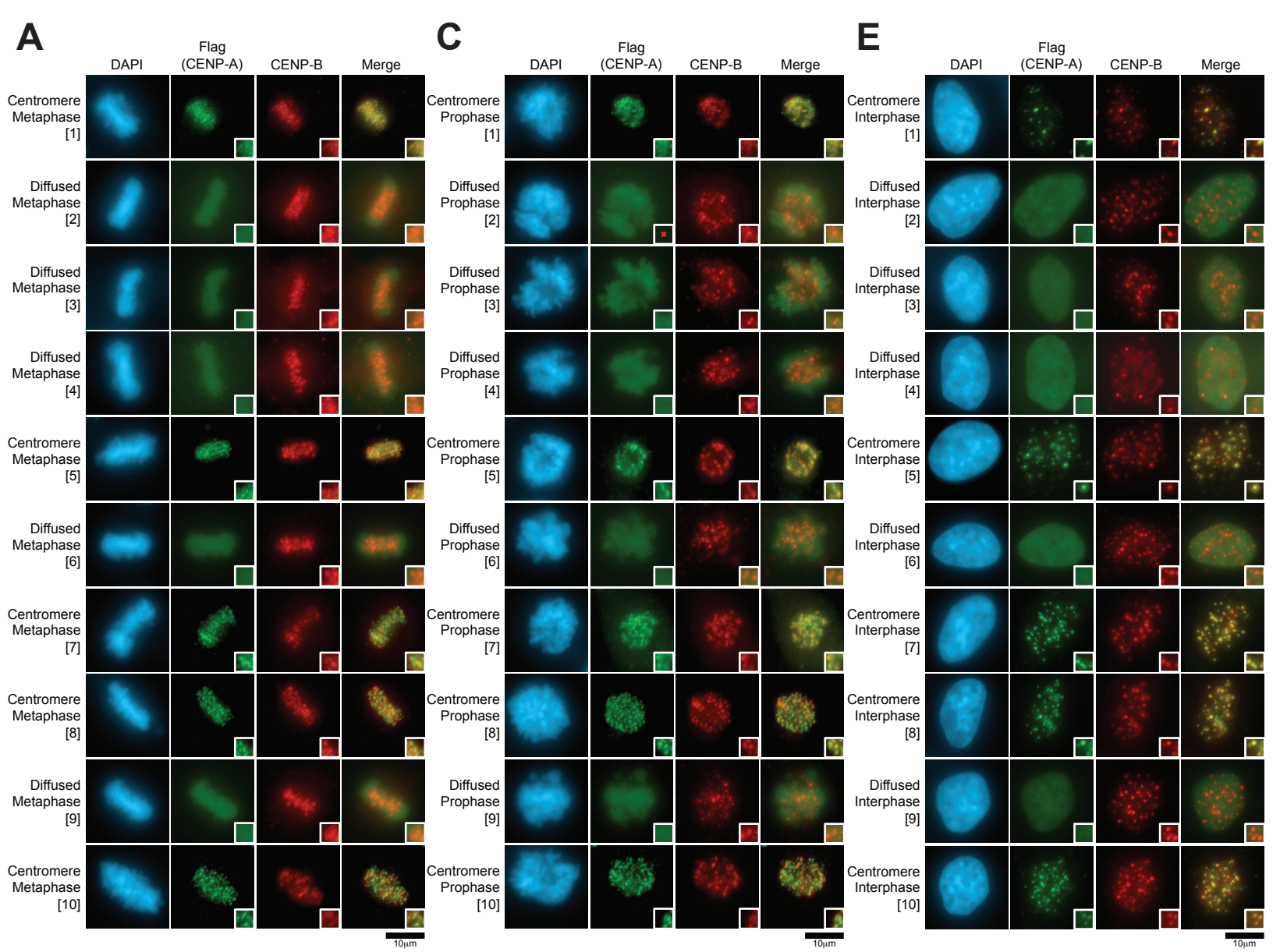
Supplementary Figure S2

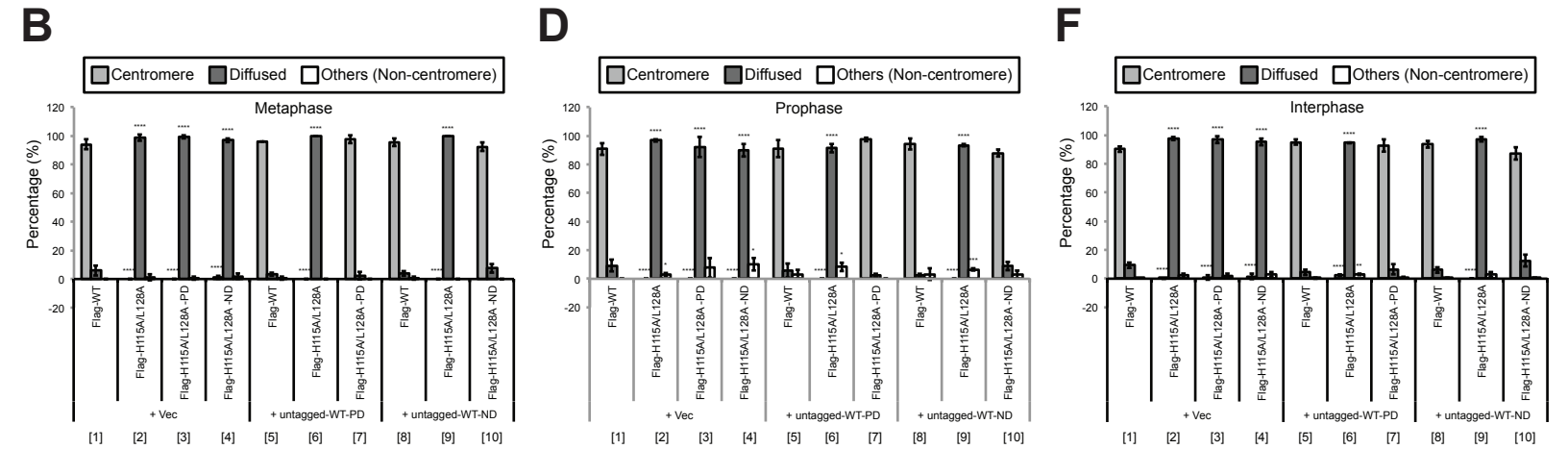


Supplementary Figure S3



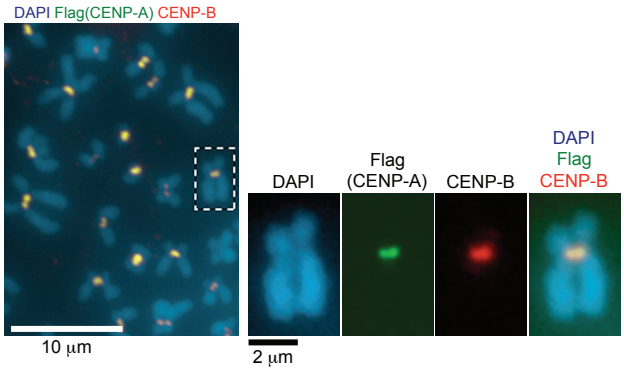
Supplementary Figure S4



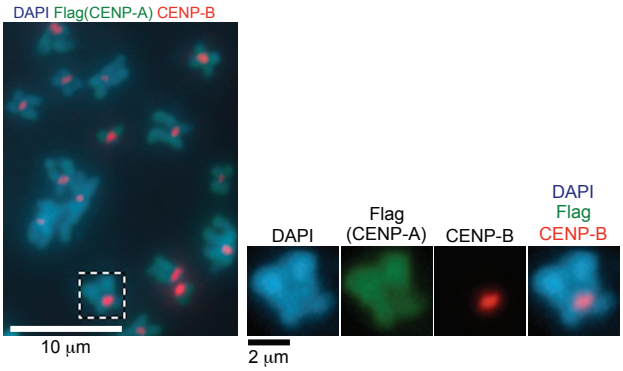


Supplementary Figure S5

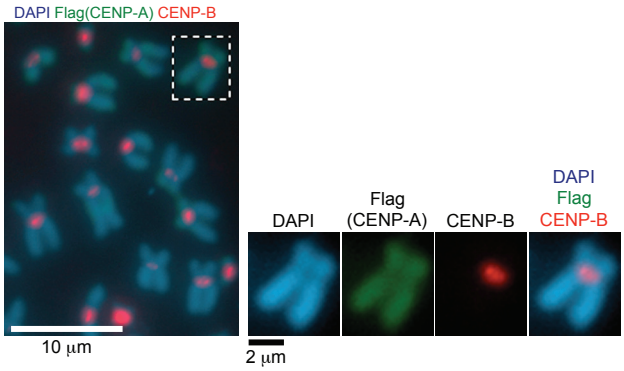
[1] Flag-WT + WT



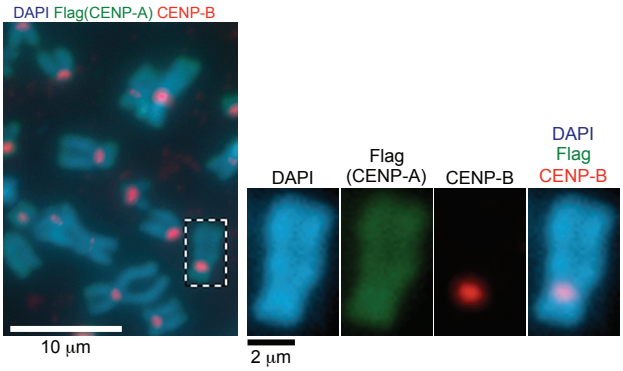
[2] Flag-H115A/L128A + WT



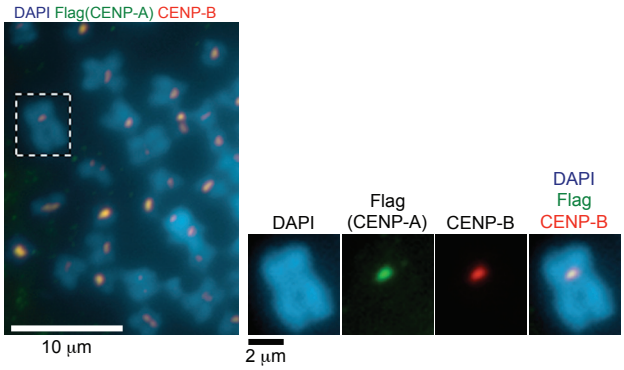
[3] Flag-H115A/L128A-PD +WT



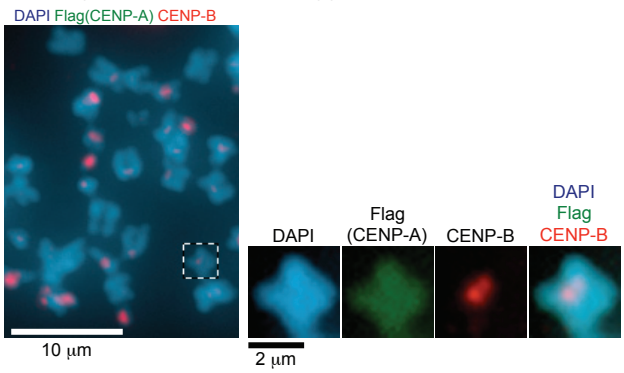
[4] Flag-H115A/L128A-ND +WT



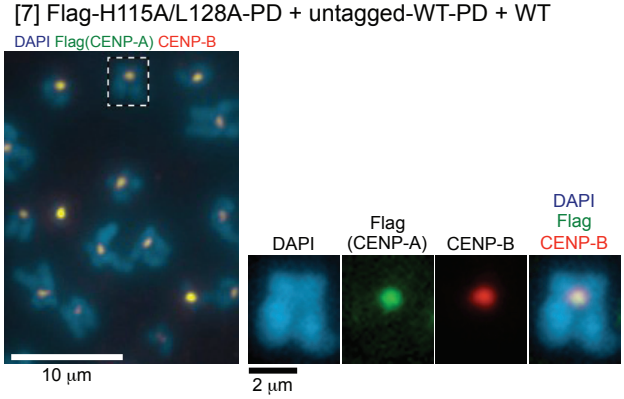
[5] Flag-WT + untagged-WT-PD + WT



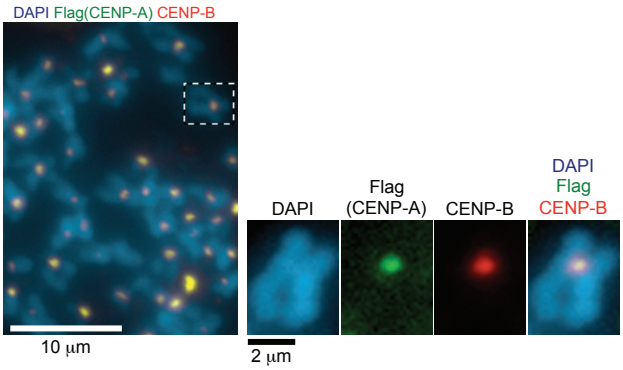
[6] Flag-H115A/L128A + untagged-WT-PD + WT



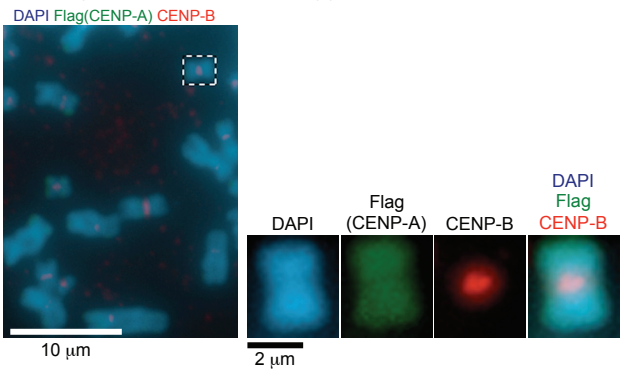
[7] Flag-H115A/L128A-PD + untagged-WT-PD + WT



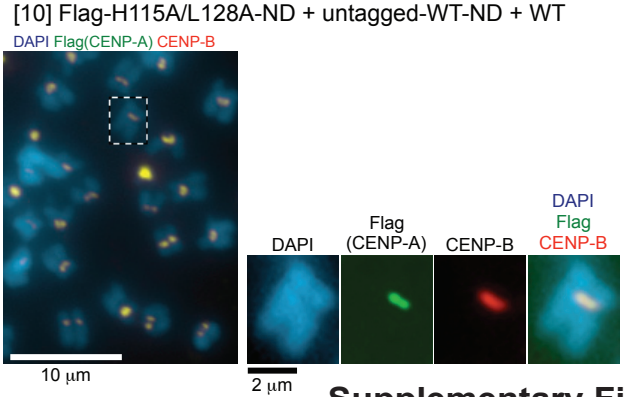
[8] Flag-WT + untagged-WT-ND + WT



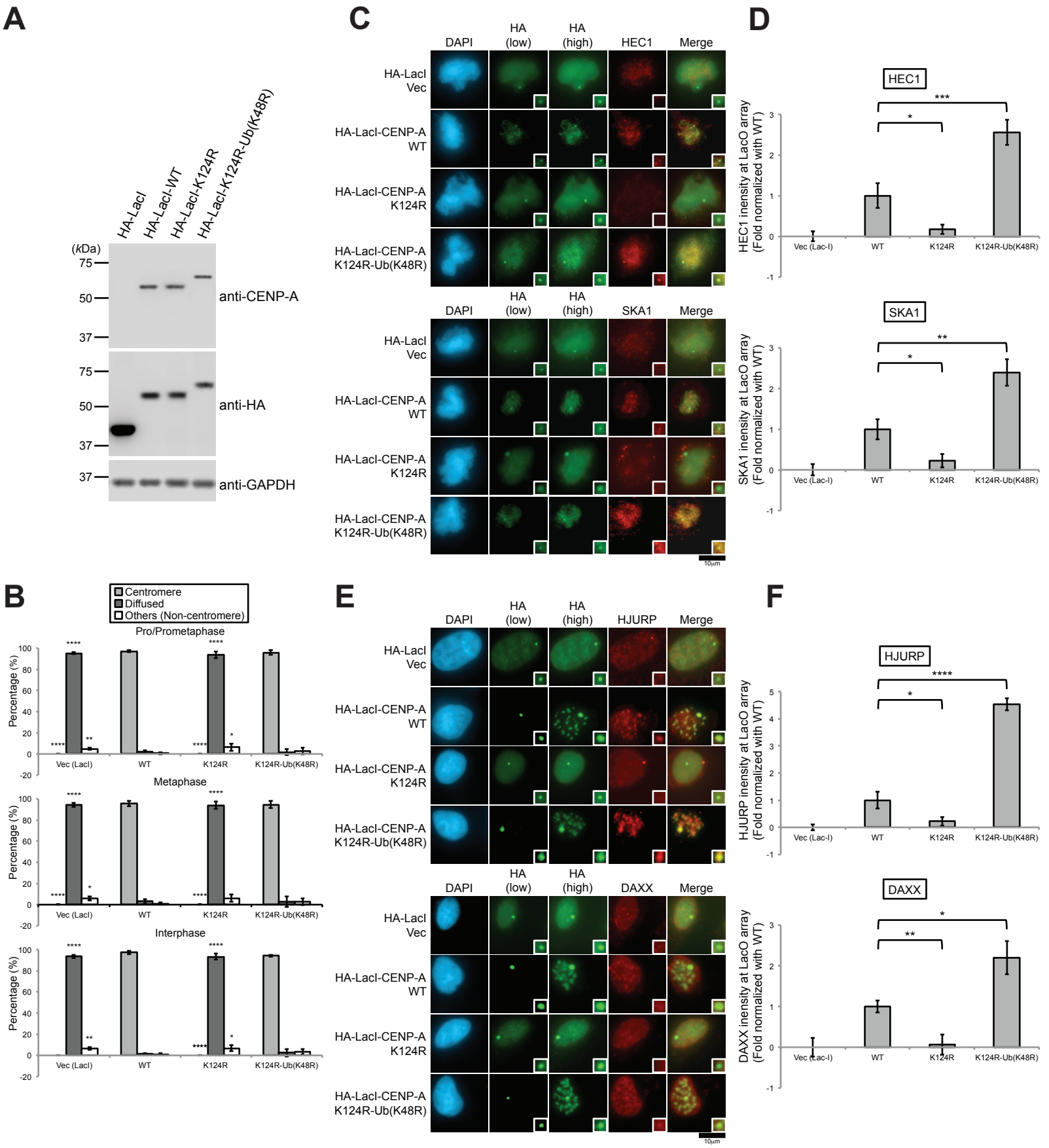
[9] Flag-H115A/L128A + untagged-WT-ND + WT



[10] Flag-H115A/L128A-ND + untagged-WT-ND + WT



Supplementary Figure S6



Supplementary Figure S7