Supplemental Information for

CENP-A K124 Ubiquitylation Is Required for CENP-A Deposition at the Centromere

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Supplemental Experimental Procedures

Immunoblotting

The method for immunoblotting has been described in detail previously (Kitagawa et al., 1999; Lamb et al., 1995). Alternatively, the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE), the Odyssey CLx Infrared imaging System (LI-COR Biosciences, Lincoln, NE), or Molecular Imager Versadoc MP4000 System (Bio-Rad, Hercules, CA) were used for detection of coimmunoblotting. Cells were suspended in denaturing buffer A (20 mM Tris-HCl pH 7.4, 50 mM NaCl, 0.5% Nonidet P-40, 0.5% deoxycholate, 0.5% SDS, 1 mM EDTA, 50 μM MG132, and complete EDTA-free protease inhibitor cocktail [Roche]) (Wang et al., 2006) or buffer used in Immunoprecipitation Assay (see next section), and 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). 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 et al., 2010; Niikura et al., 2006) with the following minor modifications. To study the C-terminal Flag-tagged CENP-A – endogenous HJURP interaction and the C-terminal Flag-tagged CENP-A – N-terminal Myc-tagged CENP-A interaction, HeLa Tet-Off cells were cultured without tetracycline/doxycycline and transfected with the indicated plasmids using Lipofectaminee 2000 (Invitrogen), Lipofectaminee 3000

(Invitrogen), or Lipofectaminee LTX (Invitrogen). At 48 h after transfection, cells were collected and dissolved in denaturing buffer C1 (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 freeze-thaw process. Proteins were immunoprecipitated with anti-Flag M2 affinity gel (SIGMA-ALDRICH) under denaturing conditions. Immunoprecipitates were washed 4 times with buffer C1, eluted with SDS sample buffer (Lamb et al., 1995), and used for Western blot analysis with the indicated antibodies. To study the N-terminal Flag-tagged exogenous CENP-A – endogenous HJURP interaction, cells were collected and immunoprecipitated as above, but buffer C2 (PBS containing 5 mM EDTA, 0.02% sodium azide, 1 mM $MgCl_2$, 50 μM MG132, and complete EDTA-free protease inhibitor cocktail [Roche]) was used for dissolving the cells and for washing. To study the C-terminal Flag-tagged CENP-A – endogenous HJURP interaction with CUL4A or RBX1 siRNA (Figure S5E), cells were cultured and cotransfected as in vivo ubiquitylation assay (see CENP-A In Vivo Ubiquitylation Assay below), and incubated with 5 μM MG132 (CALBIOCHEM) for 24 h, and denaturing buffer A (20 mM Tris-HCl pH 7.4, 50 mM NaCl, 0.5% Nonidet P-40, 0.5% deoxycholate, 0.5% SDS, 1 mM EDTA, 50 μM MG132, and complete EDTA-free protease inhibitor cocktail [Roche]) was used for dissolving the cells and for washing.

In the case that the in vitro immunoprecipitation assay was performed using purified proteins after CENP-A ubiquitylation in vitro (Figure 5B), subsequently purified GST-HJURP-6×His or GST was added to perform immunoprecipitation assay. This assay was performed in 1: 1 solution of 12.5 μl of Native Elution Buffer (See User Manual of Ni-NTA Purification System, Invitrogen, 25-0490) and 12.5 μl reaction mixture 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), 86.7 ng E2 (UbcH5c), and 10 μg ubiquitin.

RT-PCR

Total RNA from HeLa cells was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. To examine siRNA silencing activity, cDNA was synthesized from RNA (500 ng) and DNA amplification was performed using the One Step RT-PCR kit (QIAGEN, Valencia, CA). For amplification of gene-specific fragments, RT-PCR was performed under the following conditions: reverse transcription at 50 °C for 30 min, 30 cycles: 94 °C for 60 s, 50 °C for 60 s, and 72 °C for 60 s, and final extension at 72 °C for 10 min.

Immunofluorescence

The indirect immunofluorescent staining was performed as described previously (Niikura et al., 2006; Tugendreich et al., 1995; Yoda et al., 1996) with the following minor modifications. HeLa or HeLa Tet-Off cells were grown for 48 h on coverslip slides after transfection with siRNA(s) and/or overexpression plasmid vector. Approximately 1.8×10^5 cells were seeded, and cells were grown for 18 h before transfection.

To detect endogenous CENP-A proteins, HeLa or HeLa Tet-Off cells were cultured without tetracycline/doxycycline, and fixed at 48-96 h after transfection with the indicated siRNA plus vector construct. Cells were immunostained using a previously described method (Niikura et al., 2006) with the indicated antibodies (Table S3).

For staining exogenous N-terminal or C-terminal Flag-tagged CENP-A proteins, HeLa Tet-Off cells were cultured without tetracycline/doxycycline and fixed at 48 h after transfection with the indicated pTRM4-CENP-A constructs. Cells were also cotransfected with CA-UTR siRNAs (Table S4) to deplete endogenous CENP-A partially, but this partial depletion did not disrupt endogenous CENP-C localization at centromeres (data not shown). Only for prometaphase cell analysis, Taxol (10 nM) was added at 24 h after transfection, and cells were fixed at 48 h after transfection.

For C-terminal Flag-tagged CENP-A proteins, asynchronous or Taxol-treated (10 nM for 24 h) HeLa Tet-Off cells were fixed in 75% acetone at –20°C for 10 min. Cells were dried and blocked with 0.5% skim milk and 0.5% BSA in PBS at room temperature for 5 min. Cells were then incubated with a specific primary antibody for 1 h at 37°C. After cells were washed with the blocking buffer, they were incubated with the Alexa Fluor dye-conjugated secondary antibodies (Invitrogen). 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.

For N-terminal Flag-tagged CENP-A proteins, asynchronous or Taxol-treated (10 nM for 24 h) HeLa Tet-Off 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 antibody for 1 h at 37°C. After cells were washed with blocking buffer, they were incubated with the Alexa Fluor dye–conjugated secondary antibodies (Invitrogen). Slides were washed twice with blocking buffer and then incubated in TBS containing 0.1 μg/ml DAPI.

To detect pEYFP-H3-CATD chimeric proteins, cells were cotransfected with pEYFP-H3-CATD WT (generously gifted by Dr. Ben Black, Department of Biochemistry and Biophysics, Perelman School of Medicine, University of Pennsylvania; Table S5) or K125R plus CA-UTR siRNAs to deplete endogenous CENP-A partially, but this partial depletion did not disrupt endogenous CENP-C localization at centromeres (data not shown). We followed a previously described method (Black et al., 2004), but anti-GFP antibody (Table S3) was used to enhance fluorescent signals.

Cells were observed through a Leica DM IRE2 motorized fluorescence microscope equipped with an HCX PL APO 63× and 100× oil immersion lens (Leica, Bannockburn, IL), an Leica EL6000 compact light source (Leica), and an ORCA-ER high-resolution digital charge-coupled device (CCD) camera (Hamamatsu, Hamamatsu City, Japan). Image acquisition and processing including deconvolution were performed by using Openlab version 5.5.2 Scientific Imaging Software (Improvision, Lexington, MA) or Velocity version 6.1.1 3D Image Analysis Software (Improvision, Lexington, MA).

To quantify CENP-A centromere signals (CENP-A centromere remaining signals), we used a previously described method with minor modifications (Meraldi and Sorger, 2005; Niikura et al., 2006; Yang et al., 2005). The percentage of remaining signals at the centromeres was quantified by Openlab 5.5.2. Scientific Imaging Software, using the following formula:

Remaining CENP-A centromere signals

$$(\%) = \frac{\sum_{i \to n} s_{sample}(n) - b(n) / r_{sample}(n) - b(n)}{\sum_{i \to n} s_{ctrl}(n) - b(n) / r_{ctrl}(n) - b(n)} \times 100$$

where *s* is the signal brightness of the selected area, which is confirmed by CREST or CENP-B staining; *b* is the background signal brightness; r_{sample} is the reference CREST or CENP-B signals for siRNA(s)-treated cells; and r_{ctrl} is the reference CREST or CENP-B signals for Luc siRNA-treated cells. For each measurement level, at least 20 cells were used to eliminate variations in staining and image acquisition. We used CENP-B signals as reference signals for CUL4A, RBX1, DDB1, and COPS8 siRNAs, and used CREST signals for all the other siRNAs.

Exogenous CENP-A In Vivo Ubiquitylation Assay

For substrates expressed from pTRM4 vector constructs, HeLa Tet-Off cells were cultured without tetracycline/doxycycline and cotransfected with pcDNA3-HA-Ubiquitin (generously gifted by Hengbin Wang, Department of Biochemistry and Molecular Genetics, University of Alabama at Birmingham) or pCGN-HA-Ubiquitin, plus pTRM4-CENP-A (WT or K124R)-Flag, pTRM4-H3-CATD (WT or K125R), or pTRM4, and the indicated siRNA(s) using Lipofectaminee 2000 (Invitrogen), Lipofectaminee 2000 (Invitrogen), Lipofectaminee LTX (Invitrogen), or Lipofectaminee RNAiMAX (Invitrogen). At 24 h after transfection, cells were incubated with 5 μM MG132 (CALBIOCHEM) for 24 h. Cells were then collected and lysed in denaturing buffer A (20 mM Tris-HCl pH 7.4, 50 mM NaCl, 0.5% Nonidet P-40, 0.5% deoxycholate, 0.5% SDS, 1 mM EDTA, 50 μM MG132, and complete EDTA-free protease inhibitor cocktail [Roche]) by a sonication and freeze-thaw process. Proteins were immunoprecipitated with anti-Flag M2 affinity gel (SIGMA-ALDRICH) under denaturing conditions. Immunoprecipitates were washed 4 times with buffer A, and eluted with SDS sample buffer (Lamb et al., 1995). The elution was used for Western blot analysis with anti-Flag and anti-HA antibodies.

For YFP-H3-CATD WT (generously gifted by Dr. Ben Black, Department of Biochemistry and Biophysics, Perelman School of Medicine, University of Pennsylvania; Table S5) and K125R, HeLa cells were cultured, co-transfected, and lysed as described above. Proteins were immunoprecipitated with anti-GFP antibody (Table S3) under denaturing conditions, and used for Western blot analysis with the indicated antibodies.

Endogenous CENP-A In Vivo Ubiquitylation Assay

To study ubiquitylation of endogenous CENP-A in chromatin, chromatin was extracted (see Chromatin-free and Chromatin Extraction, and Subcellular Fractionation) using HeLa cells harboring a stably integrated HA-ubiquitin (Wang et al., 2006). (The stable cell line was generously gifted by Dr. Hengbin Wang, Department of Biochemistry and Molecular Genetics, University of Alabama at Birmingham.) Native chromatin immunoprecipiation (NChIP) (Izuta et al., 2006) was performed with anti-CENP-A antibody (Cα) or IgG control (IgG) (Santa Cruz) (Table S3) incubating with Protein A Sepharose CL-4B (GE Healthcare, Pittsburgh, PA) in buffer B (20 mM HEPES-NaOH pH 8.0, 300 mM NaCl, 20 mM KCl, 0.1% Nonidet P-40, 0.5 mM EDTA, 0.5 mM DTT, 0.1 mM PMSF, 0.5 μg/ml Pepstatin, 50 μM MG132, and complete EDTA-free protease inhibitor cocktail [Roche]). The immunoprecipitates were eluted with SDS sample buffer (Lamb et al., 1995) and used for Western blot analysis with the indicated antibodies. The Quick Western kit – IRDye (LI-COR Biosciences) or TrueBlot (eBioscience, San Diego, CA) was used to avoid IgG band detection.

Chromatin-free and Chromatin Extraction, and Subcellular Fractionation

Chromatin-free and Chromatin extraction was performed as described (Foltz et al., 2009; Izuta et al., 2006; Obuse et al., 2004; Yoda and Ando, 2004). Cells were homogenized by 20 strokes of a disposable pellet pestle (Kimble Chase Kontes). Subcellular fractionation was performed as described previously (Yoda and Ando, 2004) with the following minor modifications. HeLa cells expressing HA-ubiquitin were suspended in chromatin isolation buffer containing 0.1% digitonin to a final density of 2×10^7 cells/ml and homogenized with 10 strokes in a Dounce homogenizer. Nuclei were separated from the cytoplasmic fraction (1. Cytoplasm) by centrifugation at 300 *g* for 5 min. Nuclei in the pellet was suspended in HEPES buffer containing 0.3M NaCl, and the nucleoplasm fraction (2. Nucleoplasm) and chromatin fraction were separated by centrifugation at 500 *g* for 5 min. The isolated chromatin was digested with 60 U/ml of micrococcal nuclease for 5 min and then centrifuged at 13,000 *g* for 10 min to separate insoluble proteins in the pellet (3. Insoluble fraction) and soluble proteins in the supernatant (4. Soluble fraction). Proteins in each fraction were separated on 12.5% SDS-PAGE gels and transferred to a PVDF membrane. The amount of CENP-A in each fraction was determined by immunoblotting with the anti-CENP-A antibody. The position of ubiquitylated CENP-A was determined by re-blotting the same membrane with anti-HA antibody.

Protein Stability Assays

For stability assays of endogenous CENP-A, at 24 h or 48 h after transfection with siRNA(s), HeLa cells were incubated with 10 μg/ml of cycloheximide to repress protein synthesis. Cells were collected at the indicated time points and lysates were analyzed with the indicated antibodies (Table S3).

For stability assays of exogenous CENP-A-Flag, HeLa Tet-Off cells were cultured without tetracycline/doxycycline and transfected with pTRM4-CENP-A-Flag WT or KR mutants. At 24 h after transfection, transcription and translation were inhibited by adding 1 μg/ml doxycycline. Cells were collected at the indicated time points and lysates were analyzed with the indicated antibodies (Table S3).

Protein Purification and In Vitro GST Pull-Down Assay

Recombinant human 6×His-tagged COPS8 WT and ΔWD40 were expressed in BL21-Gold (DE3) bacterial cells (Stratagene/Agilent Technologies, Santa Clara, CA), using Gateway pDEST17 vector constructs (Invitrogen, see Table S5) and following manufacturer's instruction (Invitrogen/QIAGEN). Briefly, the cell pellet was sonicated in PBS containing 1% Triton X-100 and complete EDTA-free protease inhibitor cocktail (Roche), denatured in denaturing buffer (20 mM Tris-HCl pH 8.0, 8M urea, and 100 mM $NaPO_4$), and the protein fraction was run through an Ni-NTA Agarose (QIAGEN, Valencia, CA) column. Fractions were eluted with denaturing buffer at pH 6.3, 5.9, and 4.5, and protein fraction was dialyzed with PBS. For Figure 4D, recombinant human 6×His-tagged CENP-A purified from Sf9 insect cells was purchased from PROSPEC and/or MyBioSource. The quality of purification was confirmed by Ponceau P (Sigma-Aldrich)–stained Western blot membranes or Coomassie Brilliant Blue R (Sigma-Aldrich)–stained SDS-PAGE gels (Figures S2F–S2H).

Recombinant human 6×His-tagged CENP-A WT, CENP-A (K124R)-Ub (K48R), CUL4A, and RBX1 were expressed (see Baculovirus and Human Recombinant Protein Expression Using Sf9 Insect Cells), purified following manufacturer's instruction (see User Manual of Ni-NTA Purification System, Invitrogen, 25-0490), and the quality of purification was confirmed by Coomassie Brilliant Blue R (Sigma-Aldrich)–stained SDS-PAGE gels (Figure S3F).

Plasmid pGEX 6P (modified)-GST-HJURP-6×His (pDF263) was generously gifted by Dr. Daniel R. Foltz (Department of Biochemistry and Molecular Genetics, University of Virginia Medical School; Tbale S6). Recombinant human GST-HJURP-6×His was expressed and purified as described previously (Foltz et al., 2009), and the quality of purification was confirmed by SimplyBlue™ SafeStain (Figure S5H).

The in vitro GST pull-down assay was performed in buffer D (20 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM $MgCl_2$, 0.1% NP-40, 10% glycerol, 0.5% BSA, and complete EDTA-free protease inhibitor cocktail [Roche]). Recombinant proteins (GST-CENP-A, 6×HIS-COPS8, and 6×HIS-CUL4A) of Sf9 insect cell lysates were combined in buffer D and incubated for 1.5–2 h at 4°C after adding Glutathione Sepharose™ 4 FAST Flow (GE Healthcare). Glutathione Sepharose was washed 4 times in buffer D, and proteins were eluted with SDS sample buffer, separated on a 15% SDS-PAGE gel, and used for Western blot analysis with the indicated antibodies (Table S3).

In the case that in vitro GST pull-down assay was performed using purified proteins after CENP-A ubiquitylation in vitro (Figure 5A), subsequently purified GST-HJURP-6×His or GST control was added to perform in vitro GST pull-down assay. This assay was performed in 1: 1 solution of 25 µl of Native Elution Buffer (See User Manual of Ni-NTA Purification System, Invitrogen, 25-0490) and 25 µl reaction mixture 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, 200 ng E1 (UBE1), 200 ng E2 (UbcH5c), and 20 μg ubiquitin.

Baculovirus and Human Recombinant Protein Expression Using Sf9 Insect Cells

Recombinant human 6×His-tagged proteins (CENP-A WT, CENP-A K124R-Ub (K48R), CUL4A, and RBX1) 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, Table S5) to induce transposition. Recombinant bacmid DNA was selected, Sf9 insect cells were transfected with these bacmid DNA, and recombinant baculovirus particles were amplified (Table S5). Sf9 insect cells were infected with P1, P2, or higher passage baculovirus and cells were lysed in buffer E (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 10% NP-40, and complete EDTA-free protease inhibitor cocktail [Roche]) or proper lysis buffer for each assay.

In Vitro Transcription/Translation and In Vitro His Pull-Down Assay

In vitro transcription/translation of ^{35}S-labeled protein was performed according to the TNT Quick Coupled Transcription/Translation Systems protocol (Promega, Madison, WI) or TNT T7 Quick for PCR DNA protocol (Promega). PCR-generated templates of untagged CENP-A were amplified by using 5’ end primer (GAAGCTTTAATACGACTCACTATAGGGAACAGCCACCATGGGCCCGCGCCG

CCGGAGCCG) and 3’ end primer (TCACATCATCATCATCATGCCGAGTCCCTCCTCAAGGC) harboring 5×methionine (5×Met). 6×His-COPS8 was expressed in Sf9 insect cells, using the baculovirus expression system as described above. In vitro His pull-down assays were performed in buffer E (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 10% NP-40, and complete EDTA-free protease inhibitor cocktail [Roche]). Sf9 cell lysates expressing 6×His-COPS8 were combined with ^{35}S-labeled CENP-A and incubated overnight at 4°C after adding Ni-NTA Agarose (QIAGEN). Ni-NTA Agarose was washed 4 times in buffer E, and proteins were eluted from the agarose in SDS sample buffer, separated on a 15% SDS-PAGE gel, and used for Western blot analysis with the indicated antibodies. ^{35}S signals on Immobilon PVDF membranes (Millipore, Billerica, MA) were detected by X-ray film.

Mass Spectrometry

To identify the CENP-A ubiquitylation site, HeLa Tet-Off cells were cultured without tetracycline/doxycycline and cotransfected with pTRM4-human CENP-A-Flag and pcDNA3-mammalian HA-Ubiquitin. At 24 h after transfection, cells were incubated with 1 μM MG132 for 24 h and protein extracts were purified with anti-FLAG M2 affinity gel (SIGMA-ALDRICH). Samples were electrophoresed on an SDS-PAGE gel and stained with SYPRO Ruby protein gel stain (Invitrogen/Molecular Probes, Grand Island, NY). The gel region between the heavy and light chains was excised and cut into 5 bands. These protein gel bands were reduced and alkylated with dithiothreitol and iodoacetamide, respectively, and then digested with trypsin (Promega, Madison, WI) for

12 h at 37°C. The resulting peptide mixtures were pooled, acidified to pH 3.5 with formic acid, and fractionated by nanoflow reversed-phase ultrahigh-pressure liquid chromatography on a nanoAcquity ultra performance LC system (Waters Corporation, Milford, MA) and introduced online into an LTQ XL Mass Spectrometer (Thermo Fisher, San Jose, CA), using electrospray ionization (ESI). Data-dependent scanning was incorporated to select abundant precursor ions for fragmentation by acquiring a full-scan mass spectrum followed by MS/MS on the 10 most abundant ions (1 microscan per spectra; precursor $m/z \pm 1.5$ Da, 35% collision energy, 30 ms ion activation, 35 s dynamic exclusion, repeat count 2). The data were used in an automated database search against NCBInr (human), using a Mascot search routine, with the following residue modifications being allowed as static: cysteine (carbamidomethylation), and methionine (oxidation). GlyGly (K) and Phospho (STY) were also allowed as modifications as differential modifications in the search.

To identify CUL4A interactors, myc3-CUL4A was expressed in HeLa cells. (Plasmid pcDNA3-myc3-human CUL4A was gifted by Dr. Yue Xiong, Department of Biochemistry and Biophysics, Lineberger Comprehensive Cancer Center, Program in Molecular Biology and Biotechnology, University of North Carolina at Chapel Hill.) At 48 h after transfection, protein extracts were prepared and purified with anti-myc antibody. Samples were electrophoresed on an SDS-PAGE gel, stained with SYPRO Ruby protein gel stain (Invitrogen/Molecular Probes). For LS-MS/MS with the linear ion-trap method, the protein gel bands were reduced and alkylated with dithiothreitol and iodoacetamide and then digested with trypsin (Promega, Madison, WI) (12 h, 37°C). The resulting peptide mixture was acidified to pH 3.5 with formic acid and fractionated by nanoflow reversed-phase ultrahigh-pressure liquid chromatography on a nanoAcquity ultra performance LC system (Waters Corporation, Milford, MA). Tryptic peptides were loaded onto a "precolumn" (Symmetry C18, 180 μm i.d. × 20 mm, 5 μm particle; Waters Corporation), which was connected through a zero dead volume union to the analytical column (BEH C18, 75 μm i.d. × 100 mm, 1.7 μm particle; Waters Corporation). Peptides were eluted over a 86-min gradient (0%–70% B in 70 min, 70%–100% B in 86 min, where B = 70% acetonitrile, 0.2% formic acid) at a flow rate of 250 nl/min and introduced online into an LTQ Linear Ion Trap Mass Spectrometer (Thermo Fisher, San Jose, CA), using ESI. Data-dependent scanning was incorporated to select abundant precursor ions for fragmentation by acquiring a full-scan mass spectrum followed by MS/MS on the 10 most abundant ions (1 microscan per spectra; precursor *m/z* ± 1.5 Da, 35% collision energy, 30 ms ion activation, 35 s dynamic exclusion, repeat count 2). Product ions generated by fragmentation along the peptide backbone by collision-activated dissociation (b/y-type ions) were used in an automated database search against a specific database, using the Mascot search routine with following residue modifications being allowed: cysteine (carbamidomethylation) and methionine (oxidation). Database search results were verified by manual inspection of matches.

Fluorescence-Activated Cell Sorting (FACS) Analysis

Cells at each time point were washed with PBS and fixed in 4% paraformaldehyde at 4°C for 15–30 min or in 99.9% methanol at –20°C for 30 min. Cells fixed with paraformaldehyde were optionally permeabilized in PBS containing 0.5% Triton X-100 at room temperature for 10 min. Cells were stained with 10 μg/ml DAPI (Sigma-Aldrich).

DNA contents were measured by the BD LSR II flow cytometer (Applied Biosystems). Data were acquired by using the BD FACS Diva software, version 4.1.2 (BD). Data analysis was performed by using the Flowjo software, version 9.2 (Flowjo).

Yeast Two-Hybrid Assays

Gene open reading frames were cloned into Gateway two-hybrid vectors pDEST22 or pDEST32 and cotransformed into yeast host strain MaV203 (Life Technologies). Transformants were selected onto SC-Leu-Trp plates. Isolated colonies were patched onto new SC-Leu-Trp plates to create master plates. After an overnight incubation at 30°C, they were replica-plated onto SC-Leu-Trp and SC-Leu-Trp-His + 3 mM 3AT and incubated at 30°C for up to 2 days.

Ubiquitin-Mediated Fluorescence Complementation (UbFC) analysis

UbFC analysis was performed as described previously (Fang and Kerppola, 2004; Hu et al., 2002) with the following minor modifications. Plasmid pUbFC-YN173Ub was generously gifted by Dr. Tom Kerppola (Department of Biological Chemistry, University of Michigan Medical School; Table S5). Plasmid pmCherry-C2-human CENP-B as centromere marker was generously gifted by Dr. Stephan Diekmann (Leibniz Institute for Age Research, Fritz Lipmann Institute; Table S5). Other plasmids used for this assay are summarized in Table S5. To detect fluorescence emissions, cells cotransfected with plasmids encoding the indicated combinations of fusion proteins, pmCherry-C2-human CENP-B, and CA-UTR siRNAs (to deplete endogenous CENP-A partially, but this partial depletion did not disrupt endogenous CENP-C localization at centromeres [data not shown]), were incubated at 37°C for 48 h and then transferred to 30°C for 5 h or more to promote fluorophore maturation. Live image detection was started after 5 h fluorophore maturation, i.e., 53 h after transfection. Fluorescence emissions of cells were imaged as described (Fang and Kerppola, 2004; Hu et al., 2002).

Alpha assay for Kd determination

To determine Kd values of binding of GST-HJURP-6×His to 6×His-CENP-A WT, and binding of GST-HJURP-6×His to 6×His-CENP-A (K124R)-Ub (K48R), those proteins were purified (see Protein Purification and In Vitro GST Pull-Down Assay). Alpha assay was performed following manufacturer's instruction (see User's Guide to Alpha Assays Protein: Protein Interactions, PerkinElmer®) with the following conditions. Purified proteins, rabbit polyclonal anti-GST antibody, and mouse monoclonal anti-CENP-A antibody (Table S3) were mixed in 96-well white ½ AreaPlate™ (PerkinElmer®), and incubated overnight at 4°C plus 1 h at room temperature (ca. 25°C). Subsequently AlphaLISA® anti-mouse IgG Acceptor beads (AL105C; PerkinElmer®) and ANTI-Rabbit IgG Alpha Donor beads (AS105D; PerkinElmer®) were mixed and incubated for 1 h at room temperature (ca. 25°C) in dark.

SNAP-tag Pulse Chase Analysis

SNAP-tag analysis was performed as described previously (Carroll et al., 2009; Foltz et al., 2009; Jansen et al., 2007; Kevin L. Lorick, 2006; Lagana et al., 2010) with the following minor modifications. The HeLa monoclonal cell line expressing CENP-A-SNAP-3xHA (HeLa CENP-A-SNAP-3xHA) was generously gifted by Dr. Don W.

Cleveland (Ludwig Institute for Cancer Research, Department of Cell and Molecular Medicine, University of California at San Diego). HeLa CENP-A-SNAP-3×HA cells were transfected with CUL4A, RBX1, or Luc siRNA(s) 23 h or 30 h before the first thymidine release, and pulse labeled with SNAP-Cell Fluorescein followed by total CENP-A labeling with anti-HA antibody. Pulse labeling was conducted by incubating cells with the SNAP-Cell Fluorescein substrate (1.25 mM, New England Biolabs, Ipswich, MA). Cells were also blocked (SNAP-Cell Block, New England Biolabs) as indicated in the experimental schema (Figure 6A). Cells were fixed with 4% formaldehyde in PBS at room temperature for 10 min and treated with 0.1% Triton X-100 in PBS at room temperature for 10 min. Cells were then blocked with PBS containing 2% BSA and 2% FBS, and stained for total CENP-A-SNAP-3×HA by using blocking solution containing anti-HA mouse monoclonal antibody (1:100 dilution, Roche). Nucleus was stained with PBS containing 0.1 μg/ml DAPI.

Supplemental Figure Legends

Figure S1: Supplemental information related to Figure 1

(A) Overexpression of CUL4A-Flag rescues the delocalization of CENP-A when CUL4A siRNA targets 3' UTR. HeLa Tet-Off cells were cultured without tetracycline/doxycycline, and fixed at 48 h after cotransfection with siRNA (CUL4A #2: 3' UTR target or Luc) plus the plasmid construct (pTRM4-CUL4A-Flag or vector) as indicated. Note that CUL4A #2 is the same target as used in Figure 1A-1C. DAPI (blue), endogenous CENP-A (green), and endogenous CENP-B (red) were visualized. Scale bar = 10 μm.

(B) Western blot analysis of total cell lysates of HeLa Tet-Off cells in the same culture condition as (A). GAPDH protein was used as a loading control.

(C) CENP-A signals at centromeres given in (A) were quantified after sorting anti-Flag positive cells under microscope. ****$P < 0.0001$ with Luc siRNA plus vector-transfected cells (left column, Student's t-test).

(D) Overexpression of Flag-RBX1 rescues the reduction of CENPA at the centromere when RBX1 siRNA targets 3' UTR. HeLa cells were cultured and fixed at 72 h after cotransfection with siRNA (RBX1 #2: 3' UTR target or Luc) plus the plasmid construct (pcDNA3-Flag-RBX1 or vector) as indicated. DAPI (blue), endogenous CENP-A (green), and endogenous CENP-B (red) were visualized. Scale bar = 10 μm.

(E) Western blot analysis of total cell lysates of HeLa cells in the same culture condition as (D). GAPDH protein was used as a loading control.

(F) CENP-A signals at centromeres given in (D) were quantified after sorting anti-Flag positive cells under microscope. ****$P < 0.0001$ with Luc siRNA plus vector-transfected cells (left column, Student's t-test).

(G) Depletion of CUL4A and RBX1 does not affect endogenous CENP-A protein levels. Endogenous protein levels of CENP-A were measured in total HeLa cell lysates harvested 72 h after transfection with CUL4A, RBX1, CUL4A plus RBX1 siRNAs, or Luc siRNA. Beginning 48 h after transfection, cells were treated for 24 h with Taxol (+Tax) to arrest cells in mitosis or were not treated (Asyn). GAPDH protein was used as a loading control.

(H) Depletion of CUL4B, which is the closely related paralog of CUL4A, did not induce a significant reduction of CENP-A at centromeres. CUL4 has 2 closely related paralogs CUL4A and CUL4B in mammals and presents as a single gene in *S. pombe*, *Caenorhabditis elegans*, and *Drosophila melanogaster.* However, the ICEN proteins do not include CUL4B (83% identities, 91% similarities of both isoform 1 [NP_003579] and isoform 2 [NP_001073341]) (Izuta et al., 2006), and the CUL4B siRNA experiments did not show significant reduction of the CENP-A signal at centromeres (see also Figure S1I and Table S1). CENP-A signals at centromeres were assessed 72 h after transfection with CUL4B or Luc siRNA(s). Signals were normalized against Luc siRNA-treated cells, and the mean percentages (±SD) are shown. There was no significant CENP-A signal reduction in CUL4B siRNA-treated cells compared with Luc siRNA-treated cells.

(I) CUL4B mRNA was examined by RT-PCR from HeLa cells transfected with CUL4B or Luc siRNA(s) for 72 h. Reverse transcription was performed with specific primers of CUL4B (*1: Forward AGAGGGAAAGGAATGGTG; Reverse

CTGCATAGAGCCGGTTAGT; Note that CUL4B isoforms 1 and 2 have identical fragments.). Total RNA was used as a loading control.

(J) CUL4A-depleted cells showed abnormal metaphase. Abnormal metaphase was observed by DAPI staining in CUL4A or CENP-A siRNA-treated cells at a higher frequency than in Luc siRNA controls. HeLa cells were transfected for 72 h with the indicated siRNAs (see Table S4). Cells were fixed, and DNA was visualized by staining with DAPI (blue). Cytoskeletal structure was visualized by β-tubulin (green) staining and centromere location was visualized by endogenous CENP-B (red) staining. Misaligned, misaligned metaphase cell (arrows); 3 MTOC, cell with 3 microtubule-organizing center (MTOC); ≥4 MTOC, cell with 4 or more microtubule-organizing centers (MTOCs); normal, normal metaphase cell. Scale bar = 10 μm.

(K) Histogram summarizing the abnormal metaphase cells shown in (J). Mean percentages (±SD) are shown based on the summed population: Misaligned + 3 MTOC + ≥4 MTOC. Note that we observed similar morphological abnormality between CUL4A–depleted (left column) and CENP-A–depleted cells (center column). ***$P < 0.0001$ and **$P < 0.001$ compared with Luc siRNA (Student's t-test).

(L) CUL4A-depleted cells showed abnormal nuclei. Abnormal nuclei were observed by DAPI stain in CUL4A or CENP-A siRNA-treated cells at a higher frequency than in Luc siRNA controls. HeLa cells were transfected, fixed, and visualized as in (J). ≥3N, fragmented and aggregated nuclei consisting of 3 or more nuclei-like fragments; 2N, binuclei; micro, micronuclei (arrows); malformed, malformed nuclei; normal, normal-shaped nuclei; bridge, chromosome bridges (arrowheads). Scale bar = 10 μm.

(M) Histogram summarizing the abnormal nuclei shown in (L). More than 200 interphase cells were counted per experiment ($n \geq 3$ experiments), and the mean percentages (±SD) are shown based on the summed population: ≥3N + malformed + 2N + bridge + micro. Note that we observed similar morphological abnormality between CUL4A–depleted (left column) and CENP-A–depleted cells (center column). ****$P < 0.0001$ compared with Luc siRNA (Student's t-test).

Figure S2: Supplemental information related to Figure 2.

(A) Confirmation of overexpression constructs (related to Figure 2A). Western blot analysis of HeLa Tet-Off cell total lysates. Cells were cultured without tetracycline/doxycycline (-Dx) and harvested 48 h after transfection with pTRM4-CENP-A-Flag WT or pTRM4 vector. β-tubulin protein was used as a loading control.

(B) RBX1 or COPS8 siRNAs reduced ubiquitylation of CENP-A. (Top) Representative images of in vivo ubiquitylation assay with the combination of RBX1, COPS8, or Luc siRNA(s). Cell lysates were analyzed by in vivo ubiquitylation assay (see Experimental Procedures). Putative di-Ub-CENP-A-Flag (**) and putative mono-Ub-CENP-A-Flag (*) are indicated. (Middle) Confirmation of RBX1 and COPS8 knockdown. Representative images of Western blot analysis using 5% input of the same cell lysates used in experiments shown in (Top). For Western blot analysis, GAPDH protein was used as a loading control. (Bottom) Histogram shows quantified putative mono- and diubiquitylated-CENP-A-Flag bands (Ratio to Flag band signal normalized with pTRM4-CENP-A-Flag plus Luc siRNA-transfected cells [left column]). The mean percentages (±SD) are shown. ****$P < 0.0001$, ***$P < 0.001$, and **$P < 0.01$ compared with pTRM4-CENP-A-Flag plus Luc siRNA-transfected cells (left column, Student's t-test).

(C) DDB1 is not required for CENP-A recruitment to centromeres. (Top) Western blot analysis of HeLa total cell lysates harvested 72 h after transfection with DDB1 or Luc siRNA(s) (Table S4). GAPDH was used as a loading control. (Bottom) CENP-A signals at centromeres were assessed 72 h after transfection with DDB1or Luc siRNA(s). Signals were normalized with those in Luc siRNA-treated cells, and the mean percentages (±SD) are shown. There was no significant CENP-A signal reduction in DDB1 siRNA-treated cells compared with Luc siRNA-treated cells.

(D) WDR5 and EED are not required for CENP-A recruitment to centromeres. (Top) RT-PCR analysis from HeLa cells transfected with WDR5, EED, or Luc siRNA (Table S4) for 48 h. Reverse transcriptions were performed with specific primers of WDR5 (left, *1: Forward GTGTCTGGCTCAGAGGATAA; Reverse: AGCAGTCACTCTTCCACAGT) and EED (right, *1: Forward CTGGCACAGTAAAGAAGGAG; Reverse GGATGGCTCGTATTGCTATC). Total RNAs were used as loading control. (Bottom) CENP-A signals at centromeres were assessed 48 h after transfection with WDR5, EED, or Luc siRNA(s). Signals were normalized with those in Luc siRNA-treated cells, and the mean percentages (±SD) are shown. There was no significant CENP-A signal reduction in WDR5 or EED siRNA-treated cells compared with Luc siRNA-treated cells.

(E) COPS4 is not required for CENP-A recruitment to centromeres. (Top) Western blot analysis of HeLa cell total lysates harvested 48 h, 72 h, and 96 h after transfection with COPS4 or Luc siRNA(s) reveals depletion of COPS4. GAPDH protein was used as a loading control. (Bottom) CENP-A signals at centromeres were assessed 48 h, 72 h, and 96 h after transfection of HeLa cells with COPS4 or Luc siRNA(s). Signals were normalized with those in Luc siRNA-treated cells, and the mean percentages (±SD) are shown. There was no significant CENP-A signal reduction in COPS4 siRNA-treated cells compared with Luc siRNA-treated cells (Student's t-test).

(F) Purification of 6×His-CENP-A (purchased from PROSPEC) was verified by Ponceau P (Sigma-Aldrich) staining after proteins were transferred into Immobilon-FL PVDF membrane (Millipore). The indicated amount of protein was loaded onto each well. BSA was used as a quantity control. Non-specific bands are indicated (*).

(G and H) Purification of 6×His-COPS8 WT and Δ WD40 was verified by Coomassie Brilliant Blue R (Sigma-Aldrich) staining of SDS-PAGE gels. BSA was used as a quantity control. Non-specific bands are indicated (*).

(I) A specific interaction was observed between in vitro transcribed and translated ^{35}S-labeled CENP-A-5×Met and recombinant 6×His-COPS8 expressed in Sf9 insect cells (Table S5; see In Vitro Transcription/Translation and In Vitro His Pull-Down Assay in Supplemental Experimental Procedures). Proteins in 5% of the total reaction (T) and pulled down with Ni-NTA Agarose (P) were detected by Western blot analysis using the indicated antibodies, and ^{35}S signals were detected by X-ray film.

(J) Human CENP-A directly interacts with human COPS8 but not human CAND1 in yeast two-hybrid analysis (see Supplemental Experimental Procedures).

Figure S3: COPS8 interacts with CENP-A through its WD40 motif and bridges the CUL4A complex and CENP-A, and supplemental information related to Figure 2.

(A) (Top) Alignment of WD40 motifs constructed by ClustalW. UniProtKB (UP) accession numbers, GenBank™ (GB) accession numbers, or NCBI reference sequence

(REFSEQ) numbers of the sequences in the alignment are summarized in Table S6. Because COPS8 is a subunit of the COP9 signalsome (CSN), which functions as a deneddylase for CRLs, and the CRL complex interplays between substrate neddylation and recycling onto new adaptors (Pierce et al., 2013), depletion of the CSN subunits can inhibit certain processes as positive regulators of ubiquitylation. However, we identified the WD40 motif in human COPS8, terminating with phenylalanine-asparagine (F-N) instead of tryptophan-aspartic acid (W-D) in the sequential element B region, which is highly conserved with other F/Y-type WD40 proteins among different species. (Bottom) The WD40 motif consensus sequence is shown. n1 and n2 represents stretches of amino acids, variable in both sequence and length, that separate individual members of the WD40 motif and element A and B within the repeat, respectively (van der Voorn and Ploegh, 1992). Divergence of amino acids (W/F/Y) appears in the most conserved residue in the element B (arrow).

(B) COPS8 is required to bridge CUL4A and CENP-A. GST-CENP-A interacts with 6×His-CUL4A only in the presence of 6×His-COPS8 WT but not 6×His-COPS8 Δ WD40. Because the WD40 motif of the adaptor protein can recognize the substrate in CRL systems (Jackson et al., 2000), we hypothesized that COPS8 interacts with CENP-A through its WD40 motif and bridges the CUL4A complex and CENP-A as an authentic positive regulator. Indeed, we found that COPS8 bridges CUL4A and CENP-A. Proteins in 3% of the total reaction (T) and pulled down with Glutathione Sepharose (P) were detected by Western blot analysis using the indicated antibodies (see Protein Purification and In Vitro GST Pull-Down Assay in Supplemental Experimental Procedures). Spontaneously cleaved GST background bands are indicated (*). Note that deletion of the

COPS8 WD40 motif abrogated the ability of COPS8 to bridge CUL4A and CENP-A.

(C) The COPS8 WD40 motif is required to interact with CENP-A. GST-CENP-A interacts with 6×His-COPS8 WT but not with the COPS8 ΔWD40 mutant. In vitro GST pull-down assay as in (B) was performed, but 6×His-CUL4A was not added in all reactions. Spontaneously cleaved GST background bands are indicated (*).

(D) In vitro ubiquitylation assay using Sf9 cell lysates (see Experimental Procedures). 6xHis-tagged components (CUL4A, RBX1, and COPS8) are shown in the upper table. The band of putative GST-CENP-A-Ub is indicated with an arrow.

(E) Overview images of Figure 2E. Immunoblotting of anti-His is shown (bottom). The band of putative 6×His -CENP-A-Ub is indicated with an arrow.

(F) Purification of 6×His-CENP-A (WT), CENP-A 6×His-CENP-A (K124R)-Ub (K48R), 6×His-CUL4A and 6×His-RBX1 were verified by Coomassie Brilliant Blue R (Sigma-Aldrich) staining of SDS-PAGE gels. BSA was used as a quantity control.

(G) COPS8 ΔWD40 mutant abrogates ubiquitylation of CENP-A in vitro. In vitro ubiquitylation assay with the indicated components as in (D) (see Experimental Procedures). 6×His-tagged components (CLU4A, RBX1, COPS8 WT, and COPS8 ΔWD40) are shown in the left column of the upper Table. The band of putative GST-CENP-A-Ub is indicated with an arrow.

Figure S4: Structural and genetic profiles of CENP-A lysine 124 (K124) (related to Figure 3).

(A) CENP-A K124 site (red) in the crystal structure of the CENP-A nucleosome is shown. $\alpha 2$ and $\alpha 2'$ helices, $\alpha 3$ and $\alpha 3'$ helices, and loops L2 and L2' are indicated (yellow).

Histone H2A (grey), Histone H2B (silver), and DNA strand (white and light yellow) are shown. The CENP-A nucleosome structure was adapted from Tachiwana et al. (Tachiwana et al., 2011) (Protein Data Bank 3AN2). Note that that the C-terminal α2 helix, of which CATD is a major component and that harbors 5 non-conserved residues between CENP-A and H3, contributes to structural differences between CENP-A and histone H3 (Sekulic et al., 2010). The α2 helix participates by not only forming a stable $(CENP\text{-}A\text{-}H4)_2$ tetramer with its hydrophobic interactions and critical hydrogen bonding network, but also by effecting unique physical changes. The rigid interface of the α2 helix restricts solvent accessibility because of the presence of interchain contacts in the CENP-A-H4 pairs. However, the K124 residue is located in the CENP-A α3 helix, which might help to maintain both the external accessibility of E3 ligase and the CENP-A–specific kink in the α2 helix (Sekulic et al., 2010).

(B) Alignment of the C-terminal conserved region of CENP-A among different species. Ubiquitylation site on lysine 124 (K124) on human CENP-A is indicated. Conservation profiles on K124 diverge into conserved (green) and non-conserved (orange) clustering of species.

(C) Confirmation of overexpression of CENP-A-Flag constructs (WT and KR mutants) by Western blot analysis using HeLa Tet-Off total cell lysates. Cells were cultured without tetracycline/doxycycline and harvested 48 h after transfection with pTRM4-CENP-A-Flag WT, KR mutants, or pTRM4 vector. Overexpressed CENP-A-Flag was detected with anti-Flag antibody. GAPDH protein was used as a loading control. Putative CENP-A-Flag dimer (##) and CENP-A-Flag monomer (#) are indicated. Note that SDSresistant CENP-A dimers have been reported previously (Shelby et al., 1997; Yoda et al., 2000) (see also Figure S7F).

(D) Representative images of other cell cycle stages (related to Figure 3B). Note that diffused signals appear in the exogenous CENP-A-Flag overexpression, presumably because its expression level is approximately 1.0 to 1.4 orders of magnitude (10 to 25 fold) higher than endogenous CENP-A (data not shown). Scale bar = 10 μm.

(E) Histograms summarizing the localization patterns shown in (D) (related to Figure 3C). More than 50 pro/prometaphase and 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 the interphase, presumably due to transfection or other treatments. ****$P < 0.0001$, ***$P < 0.001$, **$P < 0.01$, and *$P < 0.05$ compared with CENP-A WT-Flag (Student's t-test).

(F) The K124R mutation does not stabilize the CENP-A protein**.** HeLa Tet-Off cells were cultured without tetracycline/doxycycline and transfected with pTRM4-CENP-A-Flag WT or K124R mutants. At 24 h after transfection, exogenous gene/protein expression was inhibited by doxycycline addition (this time point shown as 0 h). As a control for the inhibition of the exogenous gene/protein expression, doxycycline was added at the time of transfection (Dx). Total cell lysates were analyzed at the indicated time points for exogenous CENP-A-Flag protein levels with anti-Flag antibody. GAPDH proteins were used as loading controls.

(G) The depletion of CUL4A or RBX1 does not affect the stability of endogenous CENP-A protein. (Top) Schematic of protocol of stability assay for endogenous CENP-A protein. (Bottom) HeLa cells were transfected with CUL4A, RBX1, or Luc siRNA(s). At 24 h (left) or 48 h (right) after transfection, protein synthesis was inhibited by cycloheximide (CHX) addition (this time point shown as 0 h). Total cell lysates were analyzed for endogenous CENP-A protein levels with anti-CENP-A antibody at the indicated time points. The p21/WAF-1/Cip-1 protein was used as a control for the inhibition of protein synthesis. GAPDH protein was used as a loading control.

Figure S5: Visualization of ubiquitylated CENP-A by using the UbFC system, and supplemental information related to Figures 3 and 4.

(A) Scheme for visualization of CENP-A conjugated to ubiquitin in living cells. To directly visualize the localization of ubiquitylated CENP-A and examine the effect of the K124R mutation in living cells, we used the ubiquitin-mediated fluorescence complementation (UbFC) approach (Fang and Kerppola, 2004; Hu et al., 2002). This approach is based on complementation among fragments of fluorescent proteins when they are brought together by the covalent conjugation of ubiquitin fused to 1 fragment (designated YN) to a substrate protein (e.g., CENP-A) fused to a complementary fragment (designated CC). YN-Ub and CENP-A-CC were expressed in HeLa cells. Ubiquitin-mediated fluorescence compensation (UbFC) is achieved only when cells are transfected with both plasmids and CENP-A protein is conjugated to ubiquitin.

(B) Confirmation of expression of the constructs used in the UbFC system by Western blot analysis of HeLa cell total lysates. Cells were transfected for 53 h with pBiFC-HA-CENP-A WT-CC155 (WT-CC), pBiFC-HA-CENP-A K124R-CC155 mutant (K124R-CC), or pBiFC-HA-CC155 vector (Vector-CC). Overexpressed WT-CC and K124R-CC were detected with anti-HA antibody, but levels of CENP-A-CC remained unchanged between the WT and K124R mutant. GAPDH protein was used as a loading control.

(C) Visualization of ubiquitylated CENP-A in living cells**.** HeLa cells were cotransfected with plasmid pmCherry-C2-CENP-B plus the indicated constructs: pUbFC-HA-YN173Ub (YN-Ub), pBiFC-HA-CENP-A WT-CC155 (WT-CC), pBiFC-HA-CENP-A K124R-CC155 (K124R-CC), and pBiFC-HA-CC155 vector (Vector-CC). Fluorescent images were acquired after 53 h of transfection in living cells. Hoechst (blue), Ubiquitylated CENP-A (green) that displays fluorescence compensation, and mCherry-CENP-B (red) as a transfection and a centromere location marker were visualized. Note that UbFC at centromeres and the nuclear region occurred only when CENP-A WT-CC was conjugated with Ub-YN but not when CENP-A K124R-CC was applied (see also Figure S5D). This data shows that ubiquitylation on CENP-A lysine 124 (K124) occurs mainly at the centromeres and their vicinity (to the extent of the nuclear region; see also Discussion and Figure S7A-S7D). Scale bar = 10 μm.

(D) Histograms summarizing the percentage (%) of cells that display fluorescence compensation of ubiquitylated CENP-A among mCherry-CENP-B–positive cells that localize at centromeres as shown in (C). More than 100 living interphase cells were counted per experiment ($n \geq 3$ experiments) and the mean percentages (±SD) are shown. ****$P < 0.0001$ compared with WT-CC without YN-Ub [1st column from left] (Student's t-test).

(E) CUL4A or RBX1 siRNA reduces CENP-A interaction with HJURP in vivo. HeLa Tet-Off cells were cultured and transfected (see Immunoprecipitation Assay in Supplemental Experimental Procedures). Proteins in 3% of the total cell lysates (Input) and immunoprecipitates (IP) obtained using ANTI-FLAG M2 Affinity Gel (SIGMA-ALDRICH) were detected by immunoblotting using the indicated antibodies.

(F) Representative images of other cell cycle stages (related to Figure 4C). Scale bar = 10 μm.

(G) Histograms summarizing the localization patterns shown in (F) (related to Figure 4D). Non-fused Flag-CENP-A WT and KR mutants are also shown as controls. More than 50 pro/prometaphase and 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 the in interphase, presumably due to transfection or other treatments. ****$P < 0.0001$, ***$P < 0.001$, **$P < 0.01$, and *$P < 0.05$ compared with non-fused Flag-CENP-A K124R [column (6)] (Student’s t-test).

(H) Purification of GST-HJURP-6×His was verified by SimpleBlue^{TM} SafeStain (Life Technologies) of SDS-PAGE gels. BSA was used as a quantity control. Non-specific bands are indicated (*).

(I) Determining Kd values in a biochemical assay. Saturation curves used to determine Kd values of binding of GST-HJURP-6×His to 6×His-CENP-A (WT), and binding of GST-HJURP-6×His to 6×His-CENP-A (K124R)-Ub (K48R) in AlphaLISA$^{®}$ assay (see Supplemental Experimental Procedures). Scatchard plot (right), and Bmax and Kd values (table) are shown. Note that addition of monoubiquitin significantly reduces the Kd value of binding of GST-HJURP-6×His to 6×His-CENP-A (Kd value of binding of HJURP to WT = 6.04 nM and Kd value of binding of HJURP to K124R-Ub [K48R] = 0.80 nM).

Figure S6: Supplemental information related to Figures 6 and 7.

(A) DNA content in asynchronous HeLa CENP-A-SNAP-3XHA cells was detected by fluorescence-activated cell sorting (FACS) analysis. Cells were harvested 48 h and 72 h after transfection with CUL4A, RBX1, or Luc siRNA(s).

(B) FACS analysis profiled DNA content in HeLa CENP-A-SNAP-3XHA cells at the indicated time points after release from double thymidine–induced arrest at G1-S. Cells were transfected with CUL4A, RBX1, or Luc siRNA(s) at the times shown in Figure 6A.

(C) In vivo ubiquitylation assay using chromatin-free extracts of samples given in (D) (see Experimental Procedures). To determine the cell cycle timing of CENP-A ubiquitylation, the ubiquitylated forms of CENP-A were chased after release from a double-thymidine block. (Top) Proteins in 5% of the total chromatin-free extracts (Input) and immunoprecipitates (IP) were detected by Western blot analysis using the indicated antibodies. Putative di-Ub-CENP-A-Flag (**) and putative mono-Ub-CENP-A- Flag (*) are indicated. (Bottom) Histogram showing quantified putative mono- and diubiquitylated-CENP-A-Flag bands given in (Top). Ratio of each ubiquitylated form (in IP) to Flag band signal (in IP) was normalized with asynchronous cells (right column, Asyn), and fold differences are shown. Note that CENP-A levels increased at M/G1 in chromatin-free extracts, which is consistent with the previous reports (Foltz et al., 2009; Shelby et al., 1997), and the ubiquitylation also peaked in M/G1. As newly synthesized CENP-A is deposited at the centromere in M/G1 (Foltz et al., 2009; Jansen et al., 2007),

(D) FACS analysis profiled DNA content using samples given in (C) (see above). HeLa Tet-Off cells were transfected with pTRM4-CENP-A WT-Flag and cultured without tetracycline/doxycycline. Cells were released from a double-thymidine block, and DNA content after the release (0-12 h) was detected by FACS analysis. Cells treated with microtubule inhibitor TN-16 (TN16) and asynchronous cells (Asyn) are shown.

(E) Delocalization of H3-CATD at centromeres in response to CUL4A, RBX1, CUL4A plus RBX1, or Luc siRNA(s) treatment. HeLa cells were cotransfected for 48 h with pEYFP-H3-CATD plus with siRNA(s) targeting CUL4A, RBX1, CUL4A plus RBX1, or luciferase (Luc). Immunostaining with DAPI (blue), H3-CATD (green), and CENP-B (red) at prophase, metaphase, and interphase is shown (see Immunofluorescence in Supplemental Experimental Procedures). Scale bar = 10 μm.

(F) Histogram quantifying H3-CATD signals at centromeres given in (E). Signals were normalized with those in pEYFP-H3-CATD plus Luc siRNA-transfected cells (left column), and mean percentages (± SD) are shown. ****$P < 0.0001$ compared with pEYFP-H3-CATD plus Luc siRNA-transfected cells (left column, Student's t-test).

(G) Confirmation of overexpression of pEYFP-H3-CATD WT, and CUL4A and RBX1 knockdown by Western blot analysis using total cell lysates. HeLa cells were cultured and cotransfected as in (E). Overexpressed pEYFP-H3-CATD WT was detected with anti-GFP antibody, but levels of EYFP-H3-CATD remained unchanged in cells depleted of CUL4A, RBX1, or CUL4A plus RBX1 compared with pEYFP-H3-CATD plus Luc siRNA-transfected cells. GAPDH protein was used as a loading control. Asterisks * and ** indicate nonspecific bands.

(H) CUL4A or RBX1 siRNA abrogates ubiquitylation of H3-CATD in vivo. Cell lysates were analyzed by in vivo ubiquitylation assay (see Experimental Procedures). Proteins in 5% of the total cell lysates (Input) and immunoprecipitates (IP) obtained using anti-GFP antibody were separated by a 15% SDS-PAGE, and detected by immunoblotting using the indicated antibodies. Bands of putative di-Ub-YFP-H3-CATD (**) and putative mono-Ub-YFP-H3-CATD (*) are indicated with arrows.

Figure S7: Endogenous CENP-A is ubiquitylated in chromatin, and supplemental information related to Figure 3.

(A) In vivo ubiquitylation assay using chromatin fraction of HeLa cells harboring a stably integrated HA-ubiquitin (Wang et al., 2006). Centromeric chromatin was extracted from HeLa cells harboring a stably integrated HA-ubiquitin and native chromatin immunoprecipiation (NChIP) (Izuta et al., 2006) was performed with anti-CENP-A antibody (αCA) or IgG control (IgG) (Santa Cruz) (Table S3). Proteins in 5% of the total bulk chromatin (Input) and immunoprecipitates (IP) were detected by Western blot analysis using the indicated antibodies. Quick Western kit – IRDye (LI-COR Biosciences) or TrueBlot (eBioscience) was used to avoid IgG band detection (see Supplemental Experimental Procedures). Bands of putative endogenous mono-Ub-CENP-A (*) are indicated by arrows.

(B) Scheme for fractionation of cells to the cytoplasm fraction, nucleoplasm fraction, and insoluble and soluble chromatin fraction (see Supplemental Experimental Procedures). Each fraction number (1–4) corresponds to the lane number in (C) and (D).

(C) Distribution of endogenous CENP-A protein in fractionated samples 1–4 in (B). Proteins in each fraction were separated by a 12.5% SDS-PAGE and the endogenous CENP-A protein was detected by Western blotting with anti-CENP-A antibodies. The position of ubiquitylated CENP-A was determined by re-blotting the same membrane with anti-HA antibody.

(D) Histogram the summarizing distribution (%) of endogenous CENP-A protein in (C).

(E) CENP-A K124 site and its proximal residues might not affect directly CENP-A-HJURP interaction in the crystal structure of the HJURP-CENP-A-histone H4 complex. CENP-A lysine 124 site (red) and ribbon diagram of the HJURP-CENP-A-histone H4 complex are shown. Of the 7 residues (S68, N85, A88, Q89, L92, H104, and L112) (pink) of CENP-A that are reportedly important for appropriate interaction with HJURP (Bassett et al., 2012; Hu et al., 2011), L112 is the closest to K124. Distance between CENP-A K122: O and CENP-A L122: CD1 (5.63 Å), and between CENP-A K122: O and HJURP L18: CD2 (11.56 Å) are indicated, respectively (white, measured in VMD). Note that in the light of the minimum distance (11.56 Å) between CENP-A K124: O and HJURP L18: CD2, K124 ubiquitylation does not seem to bind to HJURP directly. Rather, K124 residue lies close to the CENP-A/CENP-A interface which is critical for the assembly of CENP-A into centromeres (Bassett et al., 2012). One may imagine that disruption of CENP-A octameric structure underlies this defect and that HJURP binding deficiencies may be caused indirectly. However, we detected that CENP-A K124R as well as WT dimerize in vivo (Figure S7F). Also, we could not find any ubiquitin interacting motif of HJURP. Thus, we speculate that monoubiquitylation might sterically affect the overall conformational change, L112 residue, or C-terminal portion of the CATD on which HJURP recognition is mainly dependent (Bassett et al., 2012). The structure was adapted from Hu et al. (Hu et al., 2011) (Protein Data Bank 3R45).

(F) CENP-A K124R dimerizes in vivo. HeLa Tet-Off cells were cultured and transfected with pcDNA3-Myc-CENP-A (WT or K124R) plus pTRM4-Flag-CENP-A (WT or K124R) or pTRM4 vector only (Table S5) (see Immunoprecipitation Assay in

Supplemental Experimental Procedures). Proteins in 3% of the total cell lysates (T) and immunoprecipitates (IP) obtained using ANTI-FLAG M2 Affinity Gel (SIGMA-ALDRICH) were detected by immunoblotting using anti-Myc antibody and anti-Flag antibody.

Table S1. CENP-A signals at centromeres after siRNA of ICEN and relevant components[1] (related to Figures 1, 2, and S2C-S2E)

siRNA	EntrezGene	siRNA mixture (see Table S4)	48 h after transfection	72 h after transfection	96 h after transfection
CUL4A	8451	#1	**Pro/Prometa; 8.2% (p<0.01)**	N.D.	N.D.
			Meta; 5.4% (p<0.0001)	N.D.	N.D.
			Int; 9.7% (p<0.0001)	N.D.	N.D.
		#2 (3' UTR) in Figure 1	**Pro/Prometa; 9.3% (p<0.001)**	N.D.	N.D.
			Meta; 9.0% (p<0.0001)	N.D.	N.D.
			Int; 6.6% (p<0.0001)	N.D.	N.D.
		#2 (3' UTR) in Figure S1	**Pro/Prometa; 12.9% (p<0.001)**	N.D.	N.D.
			Meta;6.6% (p<0.0001)	N.D.	N.D.
			Int; 4.0% (p<0.0001)	N.D.	N.D.
CUL4B	8450	#1	Pro/Prometa; No reduction	Pro/Prometa; No reduction	N.D.
			Meta; No reduction	Meta; No reduction	N.D.
			Int; N.D.	Int; No reduction	N.D.
SKP1	6500	#1	Meta; No reduction	Meta; No reduction	N.D.
		#2	Meta; No reduction	Meta; No reduction	N.D.
		#3	Meta; No reduction	Meta; No reduction	N.D.
SKP2	6502	#1	Meta; No reduction	N.D.	N.D.
		#2	Meta; No reduction	Meta; No reduction	N.D.
RBX1/ROC1	9978	#1	N.D.	**Pro/Prometa; 6.1% (p<0.0001)**	N.D.
			N.D.	**Meta; 9.0% (p<0.0001)**	N.D.
			N.D.	**Int; 5.8% (p<0.0001)**	N.D.
		#2 (3' UTR)	N.D.	**Pro/Prometa; 8.5% (p<0.001)**	N.D.
			N.D.	**Meta;4.6% (p<0.0001)**	N.D.
			N.D.	**Int; 5.6% (p<0.0001)**	N.D.
DDB1	1642	#1	Pro/Prometa; No reduction	Pro/Prometa; No reduction	N.D.
			Meta; No reduction	Meta; No reduction	N.D.
			Int; No reduction	Int; No reduction	N.D.
DDB2	1643	#1	Meta; No reduction	N.D.	N.D.
		#2	Meta; No reduction	Meta; No reduction	N.D.
WDR5	11091	#1	Pro/Prometa; No reduction	N.D.	N.D.
			Meta; No reduction	N.D.	N.D.
			Int; No reduction	N.D.	N.D.
WDR11/PHIP	55023	#1	Meta; No reduction	**Meta; 65.7% (p<0.0001)**	N.D.
EED	8726	#1	Pro/Prometa; No reduction	N.D.	N.D.

			Meta; No reduction	N.D.	N.D.
			Int; No reduction	N.D.	N.D.
RanGAP1	5905	#1	Meta; No reduction	Meta; No reduction	N.D.
RING1 / RNF1	6015	#1	Meta; No reduction	Meta; No reduction	N.D.
RING2 / RNF2	6045	#1	Meta; No reduction	Meta; No reduction	N.D.
CBLL1	79872	#1	**Meta; 36.8% (p<0.01)**	Meta; No reduction	N.D.
BMI1	648	#1	Meta; No reduction	Meta; No reduction	N.D.
RACGAP1	8467	#1	Meta; No reduction	Meta; No reduction	N.D.
KIAA1429	25962	#1	Meta; No reduction	Meta; No reduction	N.D.
ARS2	51593	#1	Meta; No reduction	Meta; No reduction	N.D.
KIF23	9493	#1	Meta; No reduction	Meta; No reduction	N.D.
SMARCA5 / SNF2h	8467	#1	**Meta; 7.0% (p<0.001)**	Meta; No reduction	N.D.
WTAP	9589	#1	**Meta; 43.6% (p<0.01)**	Meta; No reduction	N.D.
FBXW5	54461	#1	Meta; No reduction	Meta; No reduction	N.D.
HDAC-1	3065	#1	**Meta; 20.1% (p<0.05)**	Meta; No reduction	N.D.
CAND1	55832	#1	N.D.	**Pro/Prometa; 42.8% (p<0.0001)**	N.D.
			N.D.	**Meta; 39.1% (p<0.0001)**	N.D.
			N.D.	**Int; 16.6% (p<0.0001)**	N.D.
COPS8/CSN8	10920	#1	N.D.	**Pro/Prometa; 20.5% (p<0.0001)**	N.D.
			N.D.	**Meta; 24.2 (p<0.0001)**	N.D.
			N.D.	**Int; 14.8% (p<0.0001)**	N.D.
COPS4/CSN4	51138	#1	Pro/Prometa; No reduction	Pro/Prometa; No reduction	Pro/Prometa; No reduction
			Meta; No reduction	Meta; No reduction	Meta; No reduction
			Int; No reduction	Int; No reduction	Int; No reduction

[1]Bold indicates significant reduction of endogenous CENP-A signals.
[2]Note that we used CENP-B signals as reference signals for CUL4A, RBX1, DDB1, and COPS8 siRNAs, and used CREST signals for all the other siRNAs.

Table S2. Proteins identified in LC-MS/MS mass spectrometry analysis[1] (related to Figure 2)

Band number	Identified protein	Swissprot ID	MW	Peptides with expect score of $10xe^{-6}$ or less	Coverage (%)[2]
Band 1-Band 5	**CAND1_HUMAN Cullin-associated NEDD8-dissociated protein 1**	**CAND1_HUMAN**	**137999**	**11 (11)[3]**	**27**
	DDB1_HUMAN DNA damage-binding protein 1	**DDB1_HUMAN**	**128142**	**5 (6)**	**21**
	FBN1_HUMAN Fibrillin-1	FBN1_HUMAN	332682	1	0
	LAMC1_HUMAN Laminin subunit gamma-1	LAMC1_HUMAN	183191	1	1
	MYO1E_HUMAN Myosin-Ie	MYO1E_HUMAN	127552	1	2

	SMG8_HUMAN Protein SMG8	SMG8_HUMAN	110983	1	2
	PRKDC_HUMAN DNA-dependent protein kinase catalytic subunit	PRKDC_HUMAN	473749	1	0
	MTMR3_HUMAN Myotubularin-related protein 3	MTMR3_HUMAN	136157	1	1
Band 2-Band 6	**CAND1_HUMAN Cullin-associated NEDD8-dissociated protein 1**	**CAND1_HUMAN**	**137999**	**2 (2)**	**7**
	HS71L_HUMAN Heat shock 70 kDa protein 1L	HS71L_HUMAN	70730	1 (2)	12
	CUL4A_HUMAN Cullin-4A	CUL4A_HUMAN	88138	1	5
	DDX17_HUMAN Probable ATP-dependent RNA helicase	DDX17_HUMAN	72953	1	14
	RAGP1_HUMAN Ran GTPase-activating protein 1	RAGP1_HUMAN	63958	1	4
	SQSTM_HUMAN Sequestosome-1	SQSTM_HUMAN	48455	1	3
	TBA1A_HUMAN Tubulin alpha-1A chain	TBA1A_HUMAN	50788	1	4
	EWS_HUMAN RNA-binding protein EWS	EWS_HUMAN	68721	1	3
	TSP1_HUMAN Thrombospondin-1	TSP1_HUMAN	133291	1	1
	DPOD3_HUMAN DNA polymerase delta subunit 3	DPOD3_HUMAN	51653	1	2
	LKAP_HUMAN Limkain-b1	LKAP_HUMAN	195655	1	0.7
Band 3-Band 7	**COPS4/CSN4_HUMAN COP9 signalosome complex subunit 4**	**COPS4/CSN4_HUMAN**	**46525**	**7 (7)**	**53**
	COPS3/CSN3_HUMAN COP9 signalosome complex subunit 3	**COPS3/CSN3_HUMAN**	**48412**	**3 (3)**	**11**
	IMDH2_HUMAN Inosine-5'-monophosphate dehydrogenase 2	IMDH2_HUMAN	56226	1	13
	CSN2_HUMAN COP9 signalosome complex subunit 2	CSN2_HUMAN	51849	1	9
	TRI47_HUMAN Tripartite motif-containing protein 47	TRI47_HUMAN	70968	1	2
	MRI40_HUMAN BRCA1-A complex subunit MERIT40	MRI40_HUMAN	37050	1	6
	YBOX1_HUMAN Nuclease-sensitive element-binding protein 1	YBOX1_HUMAN	35903	1	12
	IMB1_HUMAN Importin subunit beta-1	IMB1_HUMAN	98420	1	1
	NADK_HUMAN NAD kinase OS=Homo sapiens	NADK_HUMAN	49881	1	3
	AS3MT_HUMAN Arsenite methyltransferase	AS3MT_HUMAN	42519	1	3
	ERLN1_HUMAN Erlin-1	ERLN1_HUMAN	39072	1	4
	SEPT2_HUMAN Septin-2 OS=Homo sapiens GN=SEPT2 PE=1 SV=1	SEPT2_HUMAN	41689	1	4
	RL14_HUMAN 60S ribosomal protein L14	RL14_HUMAN	23531	1	5
Band 4-Band 8	**COPS8/CSN8_HUMAN COP9 signalosome complex subunit 8**	**COPS8/CSN8_HUMAN**	**23268**	**4 (4)**	**26**
	TBB2C_HUMAN Tubulin beta-2C chain	TBB2C_HUMAN	50255	1	10
	TIF1B_HUMAN Transcription intermediary factor 1-beta	TIF1B_HUMAN	90261	1	4
	G3P_HUMAN Glyceraldehyde-3-phosphate dehydrogenase	G3P_HUMAN	36201	1	5
	CBX3_HUMAN Chromobox protein homolog 3	CBX3_HUMAN	20969	1	7
	TIMP2_HUMAN Metalloproteinase inhibitor 2	TIMP2_HUMAN	25067	1	6
	TKT_HUMAN Transketolase OS=Homo sapiens	TKT_HUMAN	68519	1	4
	AATM_HUMAN Aspartate aminotransferase, mitochondrial	AATM_HUMAN	47844	1	3

[1]Bold characters show identification with ≥ 2 peptides (subtracted by nonspecific peptide number) having an expected score of 10 × e-6 or less. Peptides present at a percentage less than 1% are not shown.
[2]Coverage (%) is the number of amino acids identified in peptides/total number of amino acids in the protein.
[3]Number in parentheses indicates the peptide number that has not been subtracted by the nonspecific peptide number.

Table S3. 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-Centromere (CREST sera)	Human sera	Fitzgerald Industries Intl.	90C-CS1058
Anti-CUL4A	Rabbit polyclonal	Dr. Pradip Raychaudhuri	-
Anti-RBX1	Rabbit polyclonal	Cell Signaling	4397
Anti-SGT1	Mouse monoclonal	BD	612105
Anti-SKP1	Mouse monoclonal	Abnova	H00006500-M01
Anti-HJURP	Rabbit polyclonal	Proteintech Group Inc.	15283-1-AP
Anti-HJURP	Rabbit polyclonal	SIGMA-ALDRICH	HPA008436
Anti-CAND1	Mouse monoclonal	Novus	H00055832-M01
Anti-COPS8	Mouse monoclonal	abcam	ab77462
Anti-COPS4	Goat polyclonal	Ray Biotech Inc.	ER-14-0359
Anti-DDB1	Mouse monoclonal	abcam	ab13562
Anti-GST	Rabbit polyclonal	abcam	ab9085
Anti-Flag	Mouse monoclonal	SIGMA-ALDRICH	F3165
Anti-Flag	Rabbit polyclonal	SIGMA-ALDRICH	F7425
Anti-HA	Mouse monoclonal	Roche	1666606
Anti-HA	Rabbit polyclonal	Santa Cruz	sc-805
Anti-HA	Mouse monoclonal	Covance	AFC-101P
Anti-HA	Rat monoclonal	Roche	11867431001
Anti-5XHis	Mouse monoclonal	QIAGEN	34660
Anti-myc	Mouse monoclonal	Roche	1667203

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
Anti-p21	Mouse monoclonal	Santa Cruz	sc-6246
Anti-GFP	Rabbit polyclonal	abcam	ab290
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
goat anti-rat IgG-HRP	Affinity-purified secondary antibody	Santa Cruz	sc-2006
Goat anti-Mouse IgG DyLight 549	Affinity-purified secondary antibody	Fisher Scientific	PI35507
Goat anti-Rabbit IgG DyLight 549	Affinity-purified secondary antibody	Fisher Scientific	PI35557
Goat anti-Mouse DyLight 649	Affinity-purified secondary antibody	Fisher Scientific	PI35515
Goat anti-Rabbit DyLight 649	Affinity-purified secondary antibody	Fisher Scientific	PI35565
IRDye 800CW Goat Anti-Mouse IgG	Affinity-purified secondary antibody	LI-COR Biosciences	926-32210
IRDye 800CW Goat Anti-Rabbit IgG	Affinity-purified secondary antibody	LI-COR Biosciences	926-32211
IRDye 800CW Goat Anti-Rat IgG	Affinity-purified secondary antibody	LI-COR Biosciences	926-32219
IRDye 680 Goat Anti-Mouse IgG	Affinity-purified secondary antibody	LI-COR Biosciences	926-32220
IRDye 680 Goat Anti-Rabbit IgG	Affinity-purified secondary antibody	LI-COR Biosciences	926-32221
IRDye 680 Goat Anti-Rat IgG	Affinity-purified secondary antibody	LI-COR Biosciences	926-32229
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
normal mouse IgG	Negative control IgG	Santa Cruz	SC-2025
normal rabbit IgG	Negative control IgG	Santa Cruz	SC-2027

Table S4. 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	Used in Figures S1J-S1M.	siRNA pool (8 targets mixture)	RKK77 / t1	AUUCACUCGUGGUGUGGACdTdT	this study (1)	-
			RKK79 / t2	UUGGCAAGCCCAGGCCCUAdTdT	this study (1)	-
			RKK81 / t3	GCAUUUCUAGUUCAUCUCUdTdT	this study (1)	-
			RKK109 / t4	GCAACUCGAAGAACAUCUCdTdT	Goshima et al., (2003)	
			RKK259 / t5	GCAAGAGAAAUAUGUGUUAUU	Dharmacon	M-003249-02-0005
			RKK261 / t6	UUACAUGCAGGCCGAGUUAUU	Dharmacon	M-003249-02-0005
			RKK263 / t7	GAGACAAGGUUGGCUAAAGUU	Dharmacon	M-003249-02-0005
			RKK265 / t8	CGGAGACAAGGUUGGCUAAUU	Dharmacon	M-003249-02-0005
	CA-UTR	siRNA pool (2 targets mixture)	RKK375 / 5' UTR t1	CGAGCGGCGCGGACUUCUGCCdTdT	this study (1)	
			RKK383 / 3' UTR t1	UCCUGCACCCAGUGUUUCUGUdGdT	this study (1)	
CUL4A	#1	1 target	CRKK178 / RKK309 / t1	AGCGAUCGUAAUCAAUCCUGA	QIAGEN	SI03144484
	#2 (3'UTR)	1 target	CRKK181 / RKK331 / t4	AUGCGGGUUUGAAAUAUGACA	QIAGEN	SI04370555
CUL4B	-	siRNA pool (3 targets mixture)	RKK19 / t1	UGAGGGAGAGGAGUUCAGUdTdT	this study (1)	-
			RKK21 / t2	ACCUGGCUCUGCUAAGAAGdTdT	this study (1)	-
			RKK23 / t3	AUGAUCAUGAUCAGGAGCAdTdT	this study (1)	-
SKP1	#1	siRNA pool (8 targets mixture)	RKK5 / t1	CUACUUAGACAUCAAAGGUdTdT	this study (1)	-
			RKK7 / t2	UGGAUGAUGAAGGAGAUGAdTdT	this study (1)	-
			RKK25 / t3	AGUUGACCAAGGAACACUUdTdT	this study (1)	-
			RKK27 / t4	GGAGAUGAUGACCCAGUUCdTdT	this study (1)	-
			RKK29 / t5	GCGAACAGAUGAUAUCCCUdTdT	this study (1)	-
			RKK473 / t14	GGAAAUUGCCAAACAAUCU	this study (1)	-
			RKK475 / t15	AGACCAUGUUGGAAGAUUU	this study (1)	-
			RKK477 / t16	GGAAGAUUUGGGAAUGGAU	this study (1)	-
	#2	siRNA pool (3 targets mixture)	CRKK244/RKK491 / t11	GGAACACUUUUUGAACUCAdTdT	AMBION	s12889
			CRKK245/RKK49 / t12	AACAAUCUGUGACUAUUAAdTdT	AMBION	s12890
			CRKK246/RKK495 / t13	CAAUAUCAAAAAUGACUUUdTdT	AMBION	s12891
	#3	siRNA pool (11 targets mixture)	RKK / t1	CUACUUAGACAUCAAAGGUdTdT	this study (1)	-
			RKK7 / t2	UGGAUGAUGAAGGAGAUGAdTdT	this study (1)	-
			/RKK25 / t3	AGUUGACCAAGGAACACUUdTdT	this study (1)	-
			RKK27 / t4	GGAGAUGAUGACCCAGUUCdTdT	this study (1)	-
			RKK29 / t5	GCGAACAGAUGAUAUCCCUdTdT	this study (1)	-
			CRKK244/RKK491 / t11	GGAACACUUUUUGAACUCAdTdT	AMBION	s12889
			CRKK245/RKK493 / t12	AACAAUCUGUGACUAUUAAdTdT	AMBION	s12890
			CRKK246/RKK495 / t13	CAAUAUCAAAAAUGACUUUdTdT	AMBION	s12891
			RKK473 / t14	GGAAAUUGCCAAACAAUCU	this study (1)	-
			/RKK475 / t15	AGACCAUGUUGGAAGAUUU	this study (1)	-
			RKK477 / t16	GGAAGAUUUGGGAAUGGAU	this study (1)	-
SKP2	#1	siRNA pool (4 targets mixture)	CRKK211 / t1	CAUCUAGACUUAAGUGAUA	Dharmacon	M-003324-04
			CRKK211 / t2	CUAAAGGUCCUCGGUGUUU	Dharmacon	M-003324-04
			CRKK211 / t3	GGUAUCGCCUAGCGUCUGA	Dharmacon	M-003324-04
			CRKK211 / t4	UGUCAAUACUCUCGCAAAA	Dharmacon	M-003324-04
	#2	siRNA pool (6 targets mixture)	CRKK211 / t1	CAUCUAGACUUAAGUGAUA	Dharmacon	M-003324-04
			CRKK211 / t2	CUAAAGGUCCUCGGUGUUU	Dharmacon	M-003324-04
			CRKK211 / t3	GGUAUCGCCUAGCGUCUGA	Dharmacon	M-003324-04
			CRKK211 / t4	UGUCAAUACUCUCGCAAAA	Dharmacon	M-003324-04
			CRKK212 / t5	AAGUGAUAGUGUCAUGCUUAA	QIAGEN	SI00287819

			CRKK213 / t6	ACCCUUCAACUGUUAAAGGAA	QIAGEN	SI02659692
RBX1/ROC1	#1	siRNA pool (4 targets mixture)	CRKK198 / RKK411 / t1	GAAGCGCUUUGAAGUGAAA	Dharmacon	M-004087-01
			CRKK198 / RKK413 / t2	GCAUAGAAUGUCAAGCUAA	Dharmacon	M-004087-01
			CRKK198 / RKK415 / t3	GCAAGAAGCGCUUUGAAGU	Dharmacon	M-004087-01
			CRKK198 / RKK417 / t4	CAACAGAGAGUGGGAAUUC	Dharmacon	M-004087-01
	#2 (3'UTR)	1 target	CRKK206 / RKK425 / t8	UUCCCUGCUGUUACCUAAUUA	QIAGEN	SI04438483
DDB1	-	siRNA pool (4 targets mixture)	CRKK195 / t1	GACAAGAGUUCUCAUGUUA	Dharmacon	M-012890-02
			CRKK195 / t2	GCAAGGACCUGCUGUUUAU	Dharmacon	M-012890-02
			CRKK195 / t3	UGACAUACCUUGAUAAUGG	Dharmacon	M-012890-02
			CRKK195 / t4	CAUUAGAUCGCGAUAAUAA	Dharmacon	M-012890-02
DDB2	#1	siRNA pool (4 targets mixture)	CRKK208 / t1	CAACUAGGCUGCAAGACUU	Dharmacon	M-011022-01
			CRKK208 / t2	GAUAUCAUGCUCUGGAAUU	Dharmacon	M-011022-01
			CRKK208 / t3	GACCUCCGAGAUUGUAUUA	Dharmacon	M-011022-01
			CRKK208 / t4	AGAGCGAGAUCCGAGUUUA	Dharmacon	M-011022-01
	#2	siRNA pool (6 targets mixture)	CRKK208 / t1	CAACUAGGCUGCAAGACUU	Dharmacon	M-011022-01
			CRKK208 / t2	GAUAUCAUGCUCUGGAAUU	Dharmacon	M-011022-01
			CRKK208 / t3	GACCUCCGAGAUUGUAUUA	Dharmacon	M-011022-01
			CRKK208 / t4	AGAGCGAGAUCCGAGUUUA	Dharmacon	M-011022-01
			CRKK209 / t5	AGGGAUCAAGCAGUUAUUUGA	QIAGEN	SI02664823
			CRKK210 / t6	CUGGAUUCUUACCGGAUAUUA	QIAGEN	SI02780323
WDR5	-	siRNA pool (4 targets mixture)	CRKK196 / t1	GAGAGUGGCUGGCAAGUUC	Dharmacon	M-013383-01
			CRKK196 / t2	GACGAAAGCGUGAGGAUAU	Dharmacon	M-013383-01
			CRKK196 / t3	CAGAGGAUAACCUUGUUUA	Dharmacon	M-013383-01
			CRKK196 / t4	GCACAUGACUCUCCCGUUU	Dharmacon	M-013383-01
WDR11/PHIP	-	siRNA pool (4 targets mixture)	CRKK214 / t1	GAUGGGAGGUUGUUAGCUA	Dharmacon	M-019291-00-0005
			CRKK214 / t2	CGACAUGACAAUACAGUUA	Dharmacon	M-019291-00-0005
			CRKK214 / t3	CAACACAAUUAUCGUACAA	Dharmacon	M-019291-00-0005
			CRKK214 / t4	AUAUGGAGCUUAUACCUAA	Dharmacon	M-019291-00-0005
EED	-	siRNA pool (4 targets mixture)	CRKK197 / t1	GGAAUUAUGUUGAUUGUGU	Dharmacon	M-017581-01
			CRKK197 / t2	GUACAACACUGACUCAUCA	Dharmacon	M-017581-01
			CRKK197 / t3	CAACAGAGUUACCUUGUAU	Dharmacon	M-017581-01
			CRKK197 / t4	GGAUCUAGAGGCAUAAUUA	Dharmacon	M-017581-01
RanGAP1	-	siRNA pool (4 targets mixture)	CRKK231 / t1	AAAGGUGUCAUCUGUGUUCAA	QIAGEN	SI00698376
			CRKK232 / t2	CGGCGGCCUGCCCAAGCUAAA	QIAGEN	SI04146184
			CRKK233 / t3	CUGACCGAAUGUCACCGGAAA	QIAGEN	SI04289810
			CRKK234 / t4	CUGGACGCUGCUCUUAAAGGA	QIAGEN	SI04293751
RING1/RNF1	-	siRNA pool (3 targets mixture)	CRKK216 / t1	GGUAUGUGAAGACAACUGGdTdT	AMBION	6015 / 6592
			CRKK217 / t2	GGACACGUGGCUUUUAUACdTdT	AMBION	6015 / 106899
			CRKK218 / t3	GCCAAUAAGAGGACACAAAdTdT	AMBION	6015 / 115312
RING2/RNF2	-	siRNA pool (4 targets mixture)	CRKK215 / t1	GAGGUUAGCUUUAGAAGAA	Dharmacon	M-006556-01-0005
			CRKK215 / t2	GAGAAAUACUGGAAAGUGA	Dharmacon	M-006556-01-0005
			CRKK215 / t3	GCACAGACGAGAUACAUAA	Dharmacon	M-006556-01-0005
			CRKK215 / t4	GAGAAGCAGUAUACCAUUU	Dharmacon	M-006556-01-0005
CBLL1	-	siRNA pool (4 targets mixture)	CRKK219 / t1	CAAGAUAUAGACCGUAUUA	Dharmacon	M-007069-00-0005
			CRKK219 / t2	GCAGACGAAUUCCUAUAAA	Dharmacon	M-007069-00-0005
			CRKK219 / t3	GGGAAUGAGUCCUGGUAUA	Dharmacon	M-007069-00-0005
			CRKK219 / t4	AAGUGUGGAUUGCCUAUUA	Dharmacon	M-007069-00-0005
BMI1	-	siRNA pool (4 targets mixture)	CRKK220 / t1	GAACAGAUUGGAUCGGAAA	Dharmacon	M-005230-00-0005
			CRKK220 / t2	GAGAUGGACUGACAAAUGC	Dharmacon	M-005230-00-0005
			CRKK220 / t3	GGAUAUUGCCUACAUUUAU	Dharmacon	M-005230-00-0005
			CRKK220 / t4	GAACAACGAGAAUCAAGAU	Dharmacon	M-005230-00-0005
RACGAP1	-	siRNA pool (4 targets mixture)	CRKK221 / t1	CAAAUUAUCUCUGAAGUGU	Dharmacon	M-008650-00-0005
			CRKK221 / t2	CCACAGACACCAGAUAUUA	Dharmacon	M-008650-00-0005
			CRKK221 / t3	GAACAUCAGCUUCUCAAGA	Dharmacon	M-008650-00-0005
			CRKK221 / t4	GUAAUCAGGUGGAUGUAGA	Dharmacon	M-008650-00-0005
KIAA1429	-	siRNA pool (4 targets mixture)	CRKK222 / t1	GAAUAUCGAUAACUUCUUGAUG	Dharmacon	M-019278-00-0005
			CRKK222 / t2	CAACCUAUCGCCUUAAAUG	Dharmacon	M-019278-00-0005
			CRKK222 / t3	UCUAUGAAGUCUUGUCAGA	Dharmacon	M-019278-00-0005
			CRKK222 / t4	GACGAACAGUAGACAGUAU	Dharmacon	M-019278-00-0005
ARS2	-	siRNA pool (4 targets mixture)	CRKK223 / t1	CGACCGCAGUGUUAACAUU	Dharmacon	M-019234-02-0005
			CRKK223 / t2	CAGACGAUGUUGAUUUCUU	Dharmacon	M-019234-02-0005
			CRKK223 / t3	GAUGAGGAGUGGUUUCGGU	Dharmacon	M-019234-02-0005

			CRKK223 / t4	GGAGAAUGAUCUUCGCAUC	Dharmacon	M-019234-02-0005
KIF23	-	siRNA pool (4 targets mixture)	CRKK224 / t1	CGACAUAACUUACGACAAA	Dharmacon	M-004956-01-0005
			CRKK224 / t2	GACAUGAUCUUUAACAGUA	Dharmacon	M-004956-01-0005
			CRKK224 / t3	GAACAAAUCACUAUAAGUC	Dharmacon	M-004956-01-0005
			CRKK224 / t4	GCAUAGAAGUGAUCAAUAA	Dharmacon	M-004956-01-0005
SMARCA5/SNF2h	-	siRNA pool (4 targets mixture)	CRKK225 / t1	GGAUUAAACUGGCUCAUUU	Dharmacon	M-011478-00-0005
			CRKK225 / t2	GAGGAGAUGUAAUACCUUA	Dharmacon	M-011478-00-0005
			CRKK225 / t3	GGAAUGGUAUACUCGGAUA	Dharmacon	M-011478-00-0005
			CRKK225 / t4	GGGCAAAUAGAUUCGAGUA	Dharmacon	M-011478-00-0005
WTAP	-	siRNA pool (4 targets mixture)	CRKK226 / t1	GUACACAGAUCUUAACUCU	Dharmacon	M-017323-02-0005
			CRKK226 / t2	UGGUAGACCCAGCGAUCAA	Dharmacon	M-017323-02-0005
			CRKK226 / t3	CCCAAGAAGGUUCGAUUGA	Dharmacon	M-017323-02-0005
			CRKK226 / t4	UGGAAGUUUACGCCUGAUA	Dharmacon	M-017323-02-0005
FBXW5	-	siRNA pool (4 targets mixture)	CRKK227 / t1	CAACAAGGACGACUCGCUA	Dharmacon	M-013389-01-0005
			CRKK227 / t2	GCAACGACCUGACCAUCUC	Dharmacon	M-013389-01-0005
			CRKK227 / t3	CAGCGGCCAUGUCCUGGUA	Dharmacon	M-013389-01-0005
			CRKK227 / t4	GUGUUUGGCUGUUGGCUCA	Dharmacon	M-013389-01-0005
HDAC-1	-	siRNA pool (4 targets mixture)	CRKK228 / t1	CUAAUGAGCUUCCAUACAA	Dharmacon	M-003493-02-0005
			CRKK228 / t2	GAAAGUCUGUUACUACUAC	Dharmacon	M-003493-02-0005
			CRKK228 / t3	GGACAUCGCUGUGAAUUGG	Dharmacon	M-003493-02-0005
			CRKK228 / t4	CCGGUCAUGUCCAAAGUAA	Dharmacon	M-003493-02-0005
CAND1	-	siRNA pool (4 targets mixture)	CRKK229 / RKK389 / t1	GAAAGGAGCUUAUAAGAGA	Dharmacon	M-015562-00-0005
			CRKK229 / RKK391 / t2	UAACUGAGCUGGUAAAUGU	Dharmacon	M-015562-00-0005
			CRKK229 / RKK393 / t3	GCACUAACAUGCUUUCUGA	Dharmacon	M-015562-00-0005
			CRKK229 / RKK395 / t4	GAAAUUACCAGGUUAACUA	Dharmacon	M-015562-00-0005
COPS8/CSN8	-	siRNA pool (4 targets mixture)	CRKK230 / RKK397 / t1	UAGCCAGACUGACGGAUUA	Dharmacon	M-015831-01-0005
			CRKK230 / RKK399 / t2	GAGACGGUCCAGCCAAUUA	Dharmacon	M-015831-01-0005
			CRKK230 / RKK401 / t3	AAGAAUACCACCUGCUAUA	Dharmacon	M-015831-01-0005
			CRKK230 / RKK403 / t4	GCAUAUUAGAACAAGGAUG	Dharmacon	M-015831-01-0005
COPS4/CSN4	-	siRNA pool (11 targets mixture)	CRKK247 / RKK497 / t1	GCAUCUUGCAUCUAUAUAUdTdT	AMBION	s27533
			CRKK248 / RKK499/ t2	CUUCCUAACUUGCCUGAUAdTdT	AMBION	s27534
			CRKK249 / RKK501/ t3	CGAGCAUCGUUGCUUCAGAdTdT	AMBION	s27535
			CRKK280	GAUCCAAUCACUUUGUUUC	Dharmacon	M-021037-00-0005
			CRKK280	GCACGUGUUCUUGAUUAUA	Dharmacon	M-021037-00-0005
			CRKK280	GGACGUAUGAAUGGAUUUA	Dharmacon	M-021037-00-0005
			CRKK280	GAUAGCAUCUCAAAUGAUA	Dharmacon	M-021037-00-0005
			CRKK281	AACGUGGGAUAAGCAGAUCCA	QIAGEN	SI04149649
			CRKK282	AUUGACCAGAUUGAUGGAAUA	QIAGEN	SI04179161
			CRKK283	UACUUGAAGAUUGCUAGGCUA	QIAGEN	SI04191411
			CRKK284	AAGAUAGCAUCUCAAAUGAUA	QIAGEN	SI04237394
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 S5. Plasmid/bacmid vectors used in this study (related to Experimental Procedures)

B number (B number of bacmid and/or Bv number[1])	Relevant characteristic(s)	Source	Reference
B288	pTRM4	-	(Niikura et al., 2006)/ This study
B2090	pcDNA3-myc3-human CUL4A	Dr. Yue Xiong	(Liu et al., 2002)
B2067	pTRM4-human CENP-A-Flag	-	This study
B2281	pTRM4-human CENP-A K9A-Flag	-	This study

B2387	pTRM4-human CENP-A K77R-Flag	-	This study
B2388	pTRM4-human CENP-A K124R-Flag	-	This study
B2759	pTRM4-human CUL4A-Flag	-	This study
B828	pDEST15-GST	-	This study
B2390/B2391	pDEST15-GST-human CENP-A WT	-	This study
B2593	pDEST15-GST-human CENP-A K124R	-	This study
B2069	pcDNA3-HA-Ubiquitin	Dr. Hengbin Wang / Dr. Yi Zhang	(Wang et al., 2006)
B2624	pcDNA3-Flag-human RBX1	-	This study
B1491	pcDNA3-Flag	-	This study
B2850	pcDNA3-Myc-CENP-A WT	-	This study
B2833	pcDNA3-Myc-CENP-A K124R	-	This study
B2806	pCGN-HA-Ubiquitin	-	This study
B2402/B2403	pDEST17-6xHis-human COPS4/CSN4	-	This study
B2404/B2405	pDEST17-6xHis-human COPS8/CSN8	-	This study
B2766	pDEST17-6xHis-human COPS8/CSN8 DWD40	-	This study
B2512	pTRM4-Flag-human CENP-A	-	This study
B2579	pTRM4-Flag-human CENP-A K124R	-	This study
B2513	pTRM4-Flag-human CENP-A-Ub (WT)	-	This study
B2559	pTRM4-Flag-human CENP-A K124R-Ub (WT)	-	This study
B2515	pTRM4-Flag-human CENP-A-Ub (K48R)	-	This study
B2560	pTRM4-Flag-human CENP-A K124R-Ub (K48R)	-	This study
B2681	pTRM4-H3-CATD WT	-	This study
B2685	pTRM4-H3-CATD K125R	-	This study
B2916	pDEST10-6xHis-CENP-A WT	-	This study
B2925	pDEST10-6xHis-CENP-A K124R-Ub (K48R) (1st clone)	-	This study
B2926	pDEST10-6xHis-CENP-A K124R-Ub (K48R) (2nd clone)	-	This study
B2444	pDEST10-6xHis-human CAND1	-	This study
B2436	pDEST10-6xHis-human COPS4/CSN4	-	This study
B2438	pDEST10-6xHis-human COPS8/CSN8	-	This study
B2765	pDEST10-6xHis-human COPS8/CSN8 DWD40	-	This study
B2498	pDEST10-6xHis-human CUL4A	-	This study
B2508	pDEST10-6xHis-human RBX1/ROC1	-	This study
B2408	pDEST22-human CENP-A	-	This study
B1431	pDEST32	-	This study
B2420	pDEST32-human CENP-A	-	This study
B2477	pDEST32-human CAND1	-	This study
B2478	pDEST32-human CAND1	-	This study
B2424	pDEST32-human COPS8	-	This study
B2425	pDEST32-human COPS8	-	This study
B1548	pEYFP-C1	-	This study

B2767	pEYFP-human histone H3-CATD WT	Dr. Don W. Cleveland / Dr. Ben Black	(Black et al., 2004)
B2792	pEYFP-human histone H3-CATD K125R	-	This study
B2877	pBiFC-HA-human CENP-A WT-CC155	-	This study
B2879	pBiFC-HA-human CENP-A K124R-CC155	-	This study
B2866	pBiFC-HA-CC155	Adgene (22015)	
B2426	pUbFc-YN173-HA-Ub	Dr. Tom K. Kerppola	(Fang and Kerppola, 2004; Hu et al., 2002)
B2773	pmCherry-C2-human CENP-B	Dr. Stephan Diekmann	(Hellwig et al., 2009)
B2985	pGEX 6P (modified)-GST-HJURP-6xHis (pDF263)	Dr. Daniel R. Flotz	(Barnhart et al., 2011; Foltz et al., 2009; Zasadzinska et al., 2013)
B1242 (Bv48)	pDEST20-GST	-	This study
B2434 (B3047 and B2708; Bv61 and Bv62)	pDEST20-GST-human CENP-A WT	-	This study
B2916 (B3010; Bv94)	pDEST10-6xHis-human CENP-A WT	-	This study
B2925 (B3011 and B3012; Bv98 and Bv99)	pDEST10-6xHis-human CENP-A (K124R)-Ub (K48R)	-	This study
B2444 (B2697 and B2698; Bv63 and Bv64)	pDEST10-6xHis-human CAND1	-	This study
B2436 (B2699; Bv65)	pDEST10-6xHis-human COPS4/CSN4	-	This study
B2438 (B2700 and B2701; Bv67 and Bv70)	pDEST10-6xHis-human COPS8/CSN8 WT	-	This study
B2765 (B2951; Bv84)	pDEST10-6xHis-human COPS8/CSN8 ΔWD40	-	This study
B2498 (B2704; Bv73)	pDEST10-6xHis-human CUL4A	-	This study
B2508 (B2706; Bv75)	pDEST10-6xHis-human RBX1/ROC1	-	This study

[1]B number of bacmid and/or Bv number are noted in parenthesis.

Table S6. Sequences in the alignment of WD40 motifs (related to Figures 2D and S3A)

Indication	Species	Position	Aligned sequence	UP/GB/REFSEQ number
Hs_COPS8	*Homo sapiens*	144-179	AVKGILEQGWQADSTTRMVLPRKPVAGALD VSFNKF	(UP) Q99627
Bt_COPS8	*Bos taurus*	144-179	AVKGILEQGWQADSTTRMVMPKKPVAGAL DVSFNRF	(UP) A4FV74
Gg_COPS8	*Gallus gallus*	144-177	AVKGVLEQGWQADFSTRMVMPKKPGVLDA SFNRF	(REFSEQ) NP_001041472.1
Ac_COPS8	*Anolis carolinensis*	146-182	AVKGVLDQGWQADSSTRMVMPKKPDVLGP QEPSFNRF	(UP) G1KK67
Hs_Tb	*Homo sapiens*	112-127	VACGGLDNICSIYNLK	(UP) P62873.3
Hs_Tb	*Homo sapiens*	222-256	FTGHESDINAICFFPNGNAFATGSDDATCRLF DLR	(UP) P62873.3
Hs_COR	*Homo sapiens*	242-278	MSERQVALWDTKHLEEPLSLQELDTSSGVLL PFFDPD	(UP) P31146
Dd_COR	*Dictyostelium discoideum*	168-202	VEGHSDMITSCEWNHNGSQIVTTCKDKKAR VFDPR	(GB) CAA43707.1
Dp_COR	*Dictyostelium purpureum*	181-215	IEGHSDIILSVEWNGNGSQLVTTCKDKKARV FDPR	(UP) F0ZVC2
Ce_MSI1/RBA1	*Caenorhabditis elegans*	135-151	IASRGPSDDVYIFDYTK	(UP) P90917

Sc_MSI1	*Saccharomyces cerevisiae*	145-161	IAGASSDGAIYIFDRTK	(UP) P13712
Pc_LIG	*Phanerochaete chrysosporium*	72-105	LVFHDAIAISPAMEAQGQFGGGGADGSIIIFD TI	(GB) AAA56852.1
Pc_LIG	*Phanerochaete chrysosporium*	201-231	LSAHSVAAVNDVDPTIQGLAFDSTPGIFDSQ	(GB)AAA56852.1

Table S7. Difference between observed ion and unmodified peptide mass of y-series ions (related to Figure 3A)

y-series ion	modified peptide expected mass	unmodifid peptide expected mass	Difference between modifed and unmodified peptide mass
y2	246.1561	246.1561	0.0000
y3	359.2401	359.2401	0.0000
y4	487.2987	487.2987	0.0000
y6	701.3941	701.3941	0.0000
y7	**943.4890**	**829.4461**	**114.0429**
y8	1040.5418	926.4988	114.0430
y9	1187.6102	1073.5672	114.0430
y10	1300.6943	1186.6512	114.0431

Bold shows the y-7 ion (m/z 943.4890) which confirms the modification of K124 by the di-glycine motif. The GG modification adds 114.0429 Da.

Supplemental References

Angers, S., Li, T., Yi, X., MacCoss, M.J., Moon, R.T., and Zheng, N. (2006). Molecular architecture and assembly of the DDB1-CUL4A ubiquitin ligase machinery. Nature *443*, 590-593.

Bassett, E.A., DeNizio, J., Barnhart-Dailey, M.C., Panchenko, T., Sekulic, N., Rogers, D.J., Foltz, D.R., and Black, B.E. (2012). HJURP uses distinct CENP-A surfaces to recognize and to stabilize CENP-A/histone H4 for centromere assembly. Dev Cell *22*, 749-762.

Bennett, E.J., Rush, J., Gygi, S.P., and Harper, J.W. (2010). Dynamics of cullin-RING ubiquitin ligase network revealed by systematic quantitative proteomics. Cell *143*, 951-965.

Black, B.E., Foltz, D.R., Chakravarthy, S., Luger, K., Woods, V.L., Jr., and Cleveland, D.W. (2004). Structural determinants for generating centromeric chromatin. Nature *430*, 578-582.

Bui, M., Dimitriadis, E.K., Hoischen, C., An, E., Quenet, D., Giebe, S., Nita-Lazar, A., Diekmann, S., and Dalal, Y. (2012). Cell-cycle-dependent structural transitions in the human CENP-A nucleosome in vivo. Cell *150*, 317-326.

Carroll, C.W., Silva, M.C., Godek, K.M., Jansen, L.E., and Straight, A.F. (2009). Centromere assembly requires the direct recognition of CENP-A nucleosomes by CENP-N. Nature cell biology *11*, 896-902.

Fang, D., and Kerppola, T.K. (2004). Ubiquitin-mediated fluorescence complementation reveals that Jun ubiquitinated by Itch/AIP4 is localized to lysosomes. Proc Natl Acad Sci U S A *101*, 14782-14787.

Foltz, D.R., Jansen, L.E., Bailey, A.O., Yates, J.R., 3rd, Bassett, E.A., Wood, S., Black, B.E., and Cleveland, D.W. (2009). Centromere-specific assembly of CENP-a nucleosomes is mediated by HJURP. Cell *137*, 472-484.

Han, J., Zhang, H., Zhang, H., Wang, Z., Zhou, H., and Zhang, Z. (2013). A Cul4 E3 ubiquitin ligase regulates histone hand-off during nucleosome assembly. Cell *155*, 817-829.

He, Y.J., McCall, C.M., Hu, J., Zeng, Y., and Xiong, Y. (2006). DDB1 functions as a linker to recruit receptor WD40 proteins to CUL4-ROC1 ubiquitin ligases. Genes & development *20*, 2949-2954.

Higa, L.A., Wu, M., Ye, T., Kobayashi, R., Sun, H., and Zhang, H. (2006). CUL4-DDB1 ubiquitin ligase interacts with multiple WD40-repeat proteins and regulates histone methylation. Nat Cell Biol *8*, 1277-1283.

Hu, C.D., Chinenov, Y., and Kerppola, T.K. (2002). Visualization of interactions among bZIP and Rel family proteins in living cells using bimolecular fluorescence complementation. Mol Cell *9*, 789-798.

Hu, H., Liu, Y., Wang, M., Fang, J., Huang, H., Yang, N., Li, Y., Wang, J., Yao, X., Shi, Y., *et al.* (2011). Structure of a CENP-A-histone H4 heterodimer in complex with chaperone HJURP. Genes Dev *25*, 901-906.

Izuta, H., Ikeno, M., Suzuki, N., Tomonaga, T., Nozaki, N., Obuse, C., Kisu, Y., Goshima, N., Nomura, F., Nomura, N., *et al.* (2006). Comprehensive analysis of the ICEN (Interphase Centromere Complex) components enriched in the CENP-A chromatin of human cells. Genes Cells *11*, 673-684.

Jackson, P.K., Eldridge, A.G., Freed, E., Furstenthal, L., Hsu, J.Y., Kaiser, B.K., and Reimann, J.D. (2000). The lore of the RINGs: substrate recognition and catalysis by ubiquitin ligases. Trends Cell Biol *10*, 429-439.

Jackson, S., and Xiong, Y. (2009). CRL4s: the CUL4-RING E3 ubiquitin ligases. Trends in biochemical sciences *34*, 562-570.

Jansen, L.E., Black, B.E., Foltz, D.R., and Cleveland, D.W. (2007). Propagation of centromeric chromatin requires exit from mitosis. J Cell Biol *176*, 795-805.

Jin, J., Arias, E.E., Chen, J., Harper, J.W., and Walter, J.C. (2006). A family of diverse Cul4-Ddb1-interacting proteins includes Cdt2, which is required for S phase destruction of the replication factor Cdt1. Mol Cell *23*, 709-721.

Kevin L. Lorick, Y.Y., Jane P. Jensen, Kazuhiro Iwai, and Allan. M. Weissman (2006). Detection of E3 Activity in Immunoprecipitated Protein. In Current Protocol in Cell Biology (John Wiley & Sons, Inc.), pp. 15.19.25-15.19.26.

Kitagawa, K., Skowyra, D., Elledge, S.J., Harper, J.W., and Hieter, P. (1999). SGT1 encodes an essential component of the yeast kinetochore assembly pathway and a novel subunit of the SCF ubiquitin ligase complex. Mol Cell *4*, 21-33.

Lagana, A., Dorn, J.F., De Rop, V., Ladouceur, A.M., Maddox, A.S., and Maddox, P.S. (2010). A small GTPase molecular switch regulates epigenetic centromere maintenance by stabilizing newly incorporated CENP-A. Nature cell biology *12*, 1186-1193.

Lamb, J.R., Tugendreich, S., and Hieter, P. (1995). Tetratrico peptide repeat interactions: to TPR or not to TPR? Trends Biochem Sci *20*, 257-259.

Meraldi, P., and Sorger, P.K. (2005). A dual role for Bub1 in the spindle checkpoint and chromosome congression. EMBO J *24*, 1621-1633.

Niikura, Y., Dixit, A., Scott, R., Perkins, G., and Kitagawa, K. (2007). BUB1 mediation of caspase-independent mitotic death determines cell fate. J Cell Biol *178*, 283-296.

Niikura, Y., Ogi, H., Kikuchi, K., and Kitagawa, K. (2010). BUB3 that dissociates from BUB1 activates caspase-independent mitotic death (CIMD). Cell Death Differ *17*, 1011-1024.

Niikura, Y., Ohta, S., Vandenbeldt, K.J., Abdulle, R., McEwen, B.F., and Kitagawa, K. (2006). 17-AAG, an Hsp90 inhibitor, causes kinetochore defects: a novel mechanism by which 17-AAG inhibits cell proliferation. Oncogene *25*, 4133-4146.

Obuse, C., Yang, H., Nozaki, N., Goto, S., Okazaki, T., and Yoda, K. (2004). Proteomics analysis of the centromere complex from HeLa interphase cells: UV-damaged DNA binding protein 1 (DDB-1) is a component of the CEN-complex, while BMI-1 is transiently co-localized with the centromeric region in interphase. Genes Cells *9*, 105-120.

Pierce, N.W., Lee, J.E., Liu, X., Sweredoski, M.J., Graham, R.L., Larimore, E.A., Rome, M., Zheng, N., Clurman, B.E., Hess, S., *et al.* (2013). Cand1 promotes assembly of new SCF complexes through dynamic exchange of F box proteins. Cell *153*, 206-215.

Sekulic, N., Bassett, E.A., Rogers, D.J., and Black, B.E. (2010). The structure of (CENP-A-H4)(2) reveals physical features that mark centromeres. Nature *467*, 347-351.

Shelby, R.D., Vafa, O., and Sullivan, K.F. (1997). Assembly of CENP-A into centromeric chromatin requires a cooperative array of nucleosomal DNA contact sites. J Cell Biol *136*, 501-513.

Tachiwana, H., Kagawa, W., Shiga, T., Osakabe, A., Miya, Y., Saito, K., Hayashi-Takanaka, Y., Oda, T., Sato, M., Park, S.Y., *et al.* (2011). Crystal structure of the human centromeric nucleosome containing CENP-A. Nature *476*, 232-235.

Tatsumi, Y., Ohta, S., Kimura, H., Tsurimoto, T., and Obuse, C. (2003). The ORC1 cycle in human cells: I. cell cycle-regulated oscillation of human ORC1. J Biol Chem *278*, 41528-41534.

Tugendreich, S., Tomkiel, J., Earnshaw, W., and Hieter, P. (1995). CDC27Hs colocalizes with CDC16Hs to the centrosome and mitotic spindle and is essential for the metaphase to anaphase transition. Cell *81*, 261-268.

van der Voorn, L., and Ploegh, H.L. (1992). The WD-40 repeat. FEBS Lett *307*, 131-134.

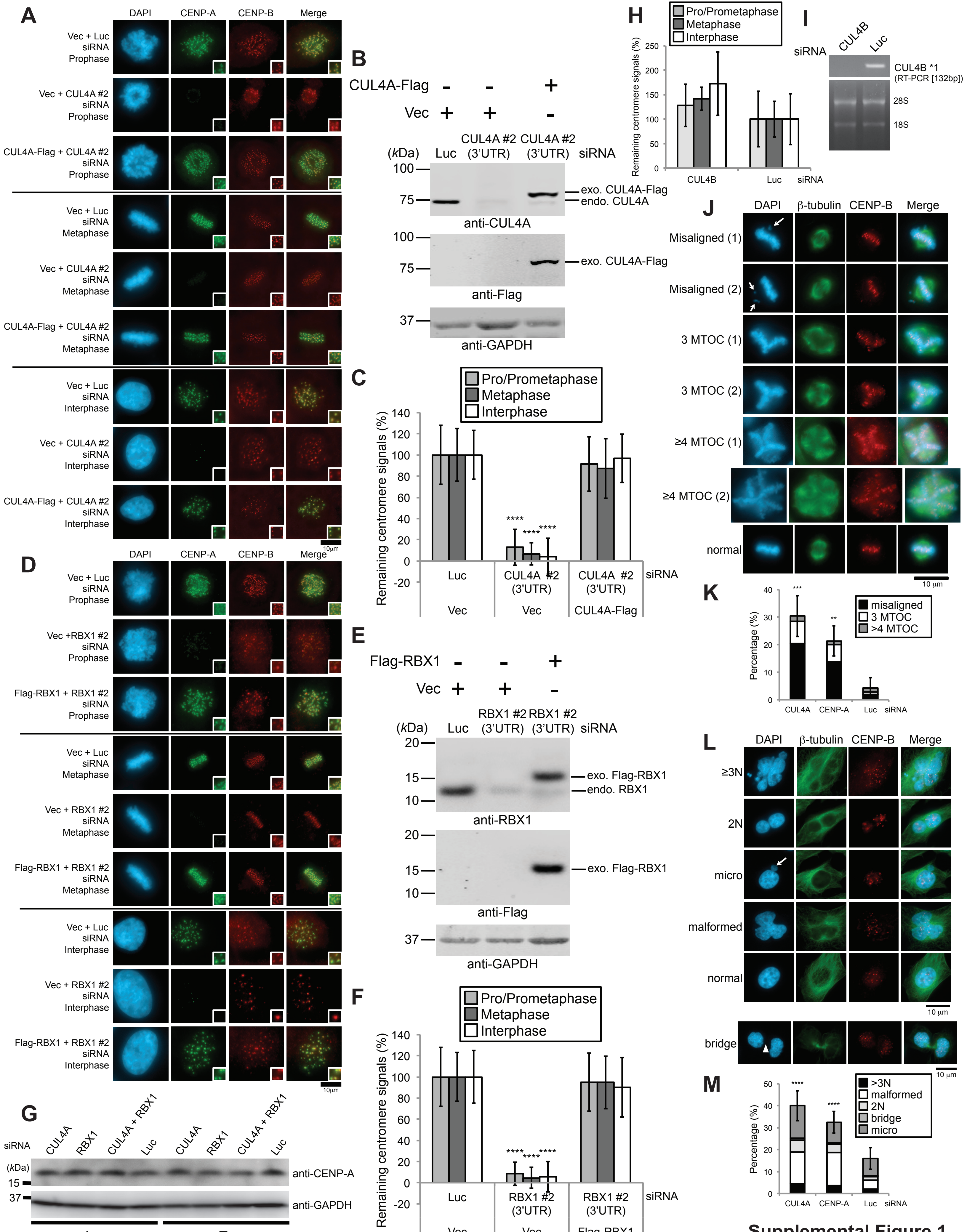
Wang, H., Zhai, L., Xu, J., Joo, H.Y., Jackson, S., Erdjument-Bromage, H., Tempst, P., Xiong, Y., and Zhang, Y. (2006). Histone H3 and H4 ubiquitylation by the CUL4-DDB-ROC1 ubiquitin ligase facilitates cellular response to DNA damage. Mol Cell *22*, 383-394.

Yang, Z., Guo, J., Chen, Q., Ding, C., Du, J., and Zhu, X. (2005). Silencing mitosin induces misaligned chromosomes, premature chromosome decondensation before anaphase onset, and mitotic cell death. Mol Cell Biol *25*, 4062-4074.

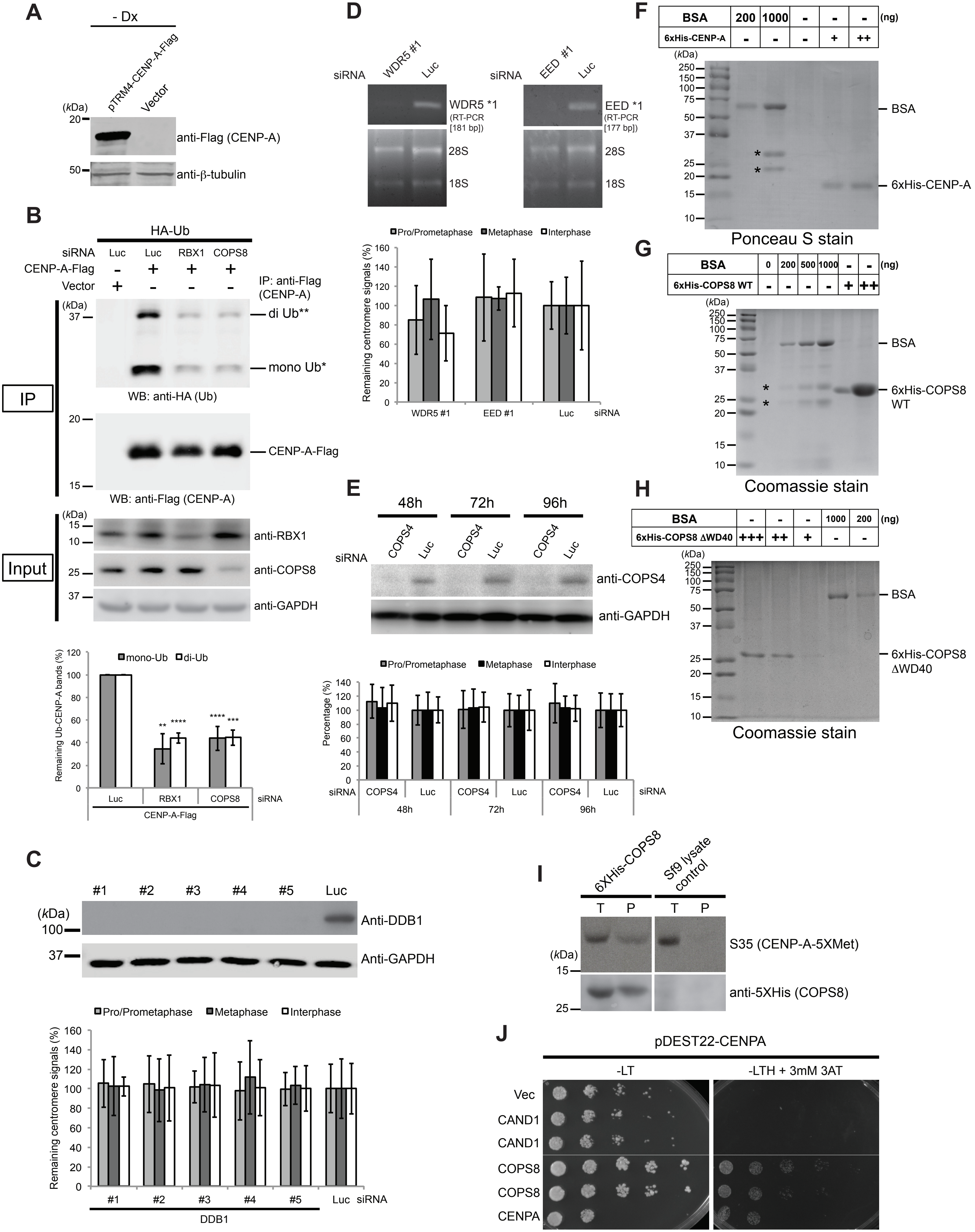
Yoda, K., and Ando, S. (2004). Immunological analysis and purification of centromere complex. Methods Enzymol *375*, 270-277.

Yoda, K., Ando, S., Morishita, S., Houmura, K., Hashimoto, K., Takeyasu, K., and Okazaki, T. (2000). Human centromere protein A (CENP-A) can replace histone H3 in nucleosome reconstitution in vitro. Proc Natl Acad Sci U S A *97*, 7266-7271.

Yoda, K., Nakamura, T., Masumoto, H., Suzuki, N., Kitagawa, K., Nakano, M., Shinjo, A., and Okazaki, T. (1996). Centromere protein B of African green monkey cells: gene structure, cellular expression, and centromeric localization. Mol Cell Biol *16*, 5169-5177.



Supplemental Figure 1



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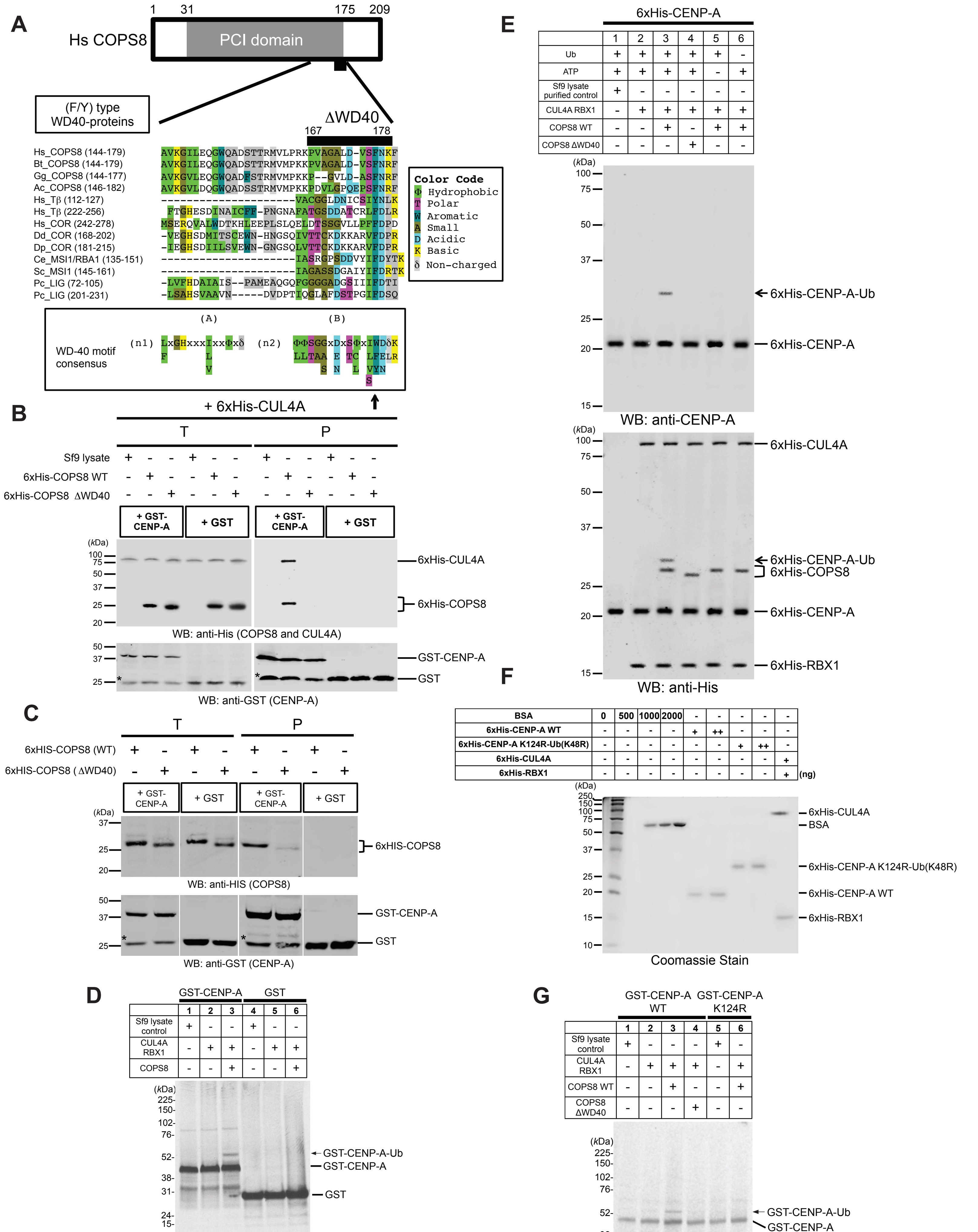

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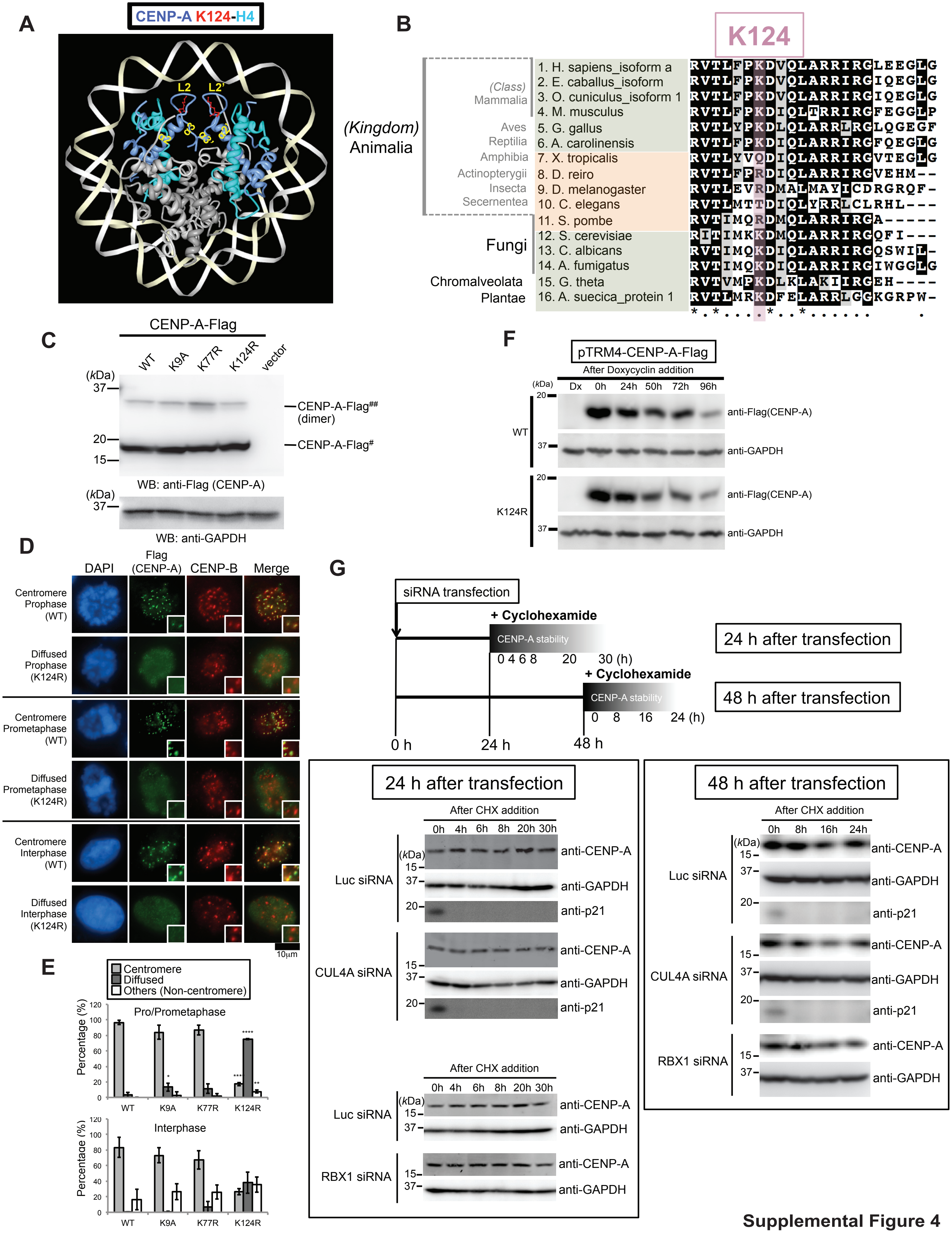
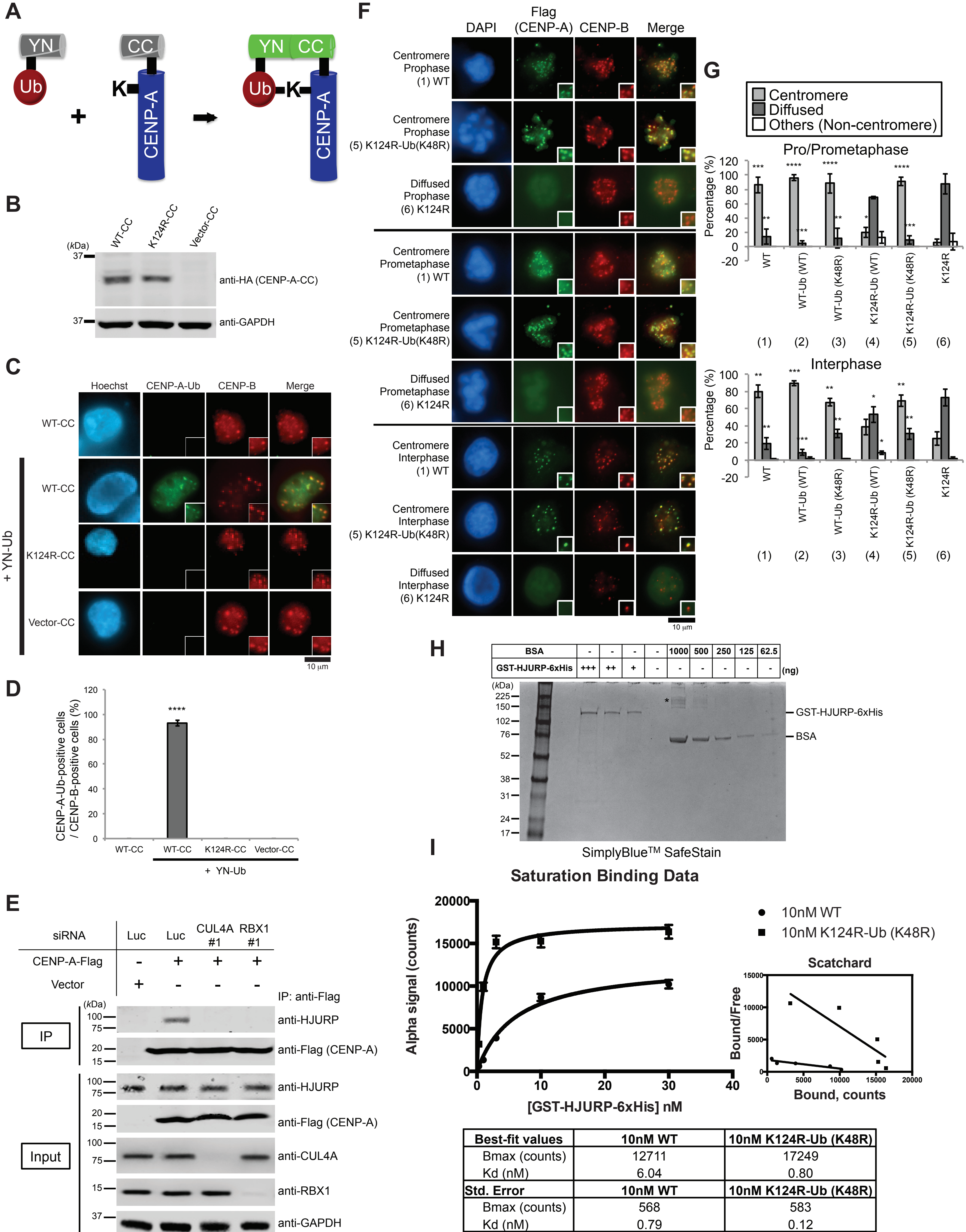
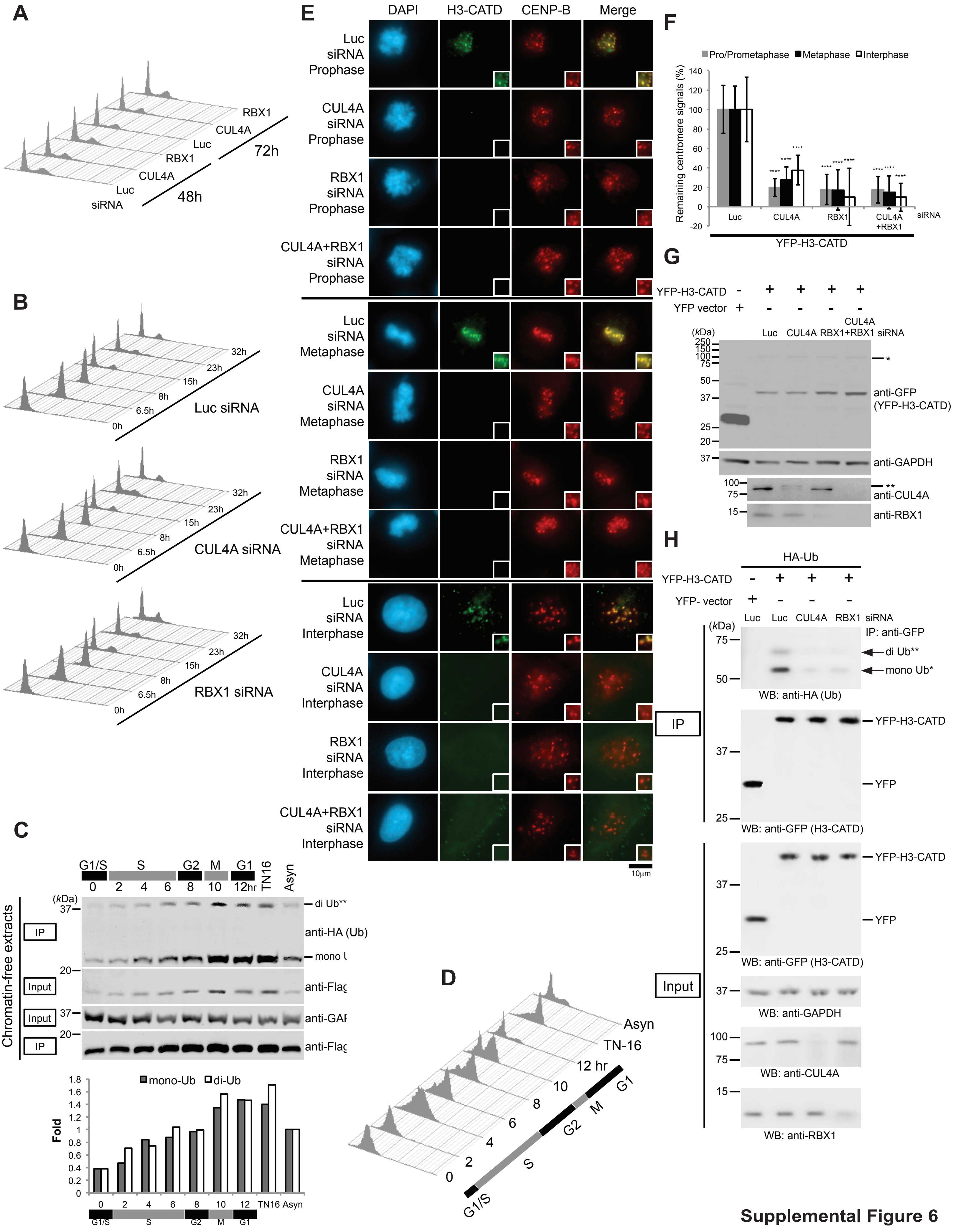


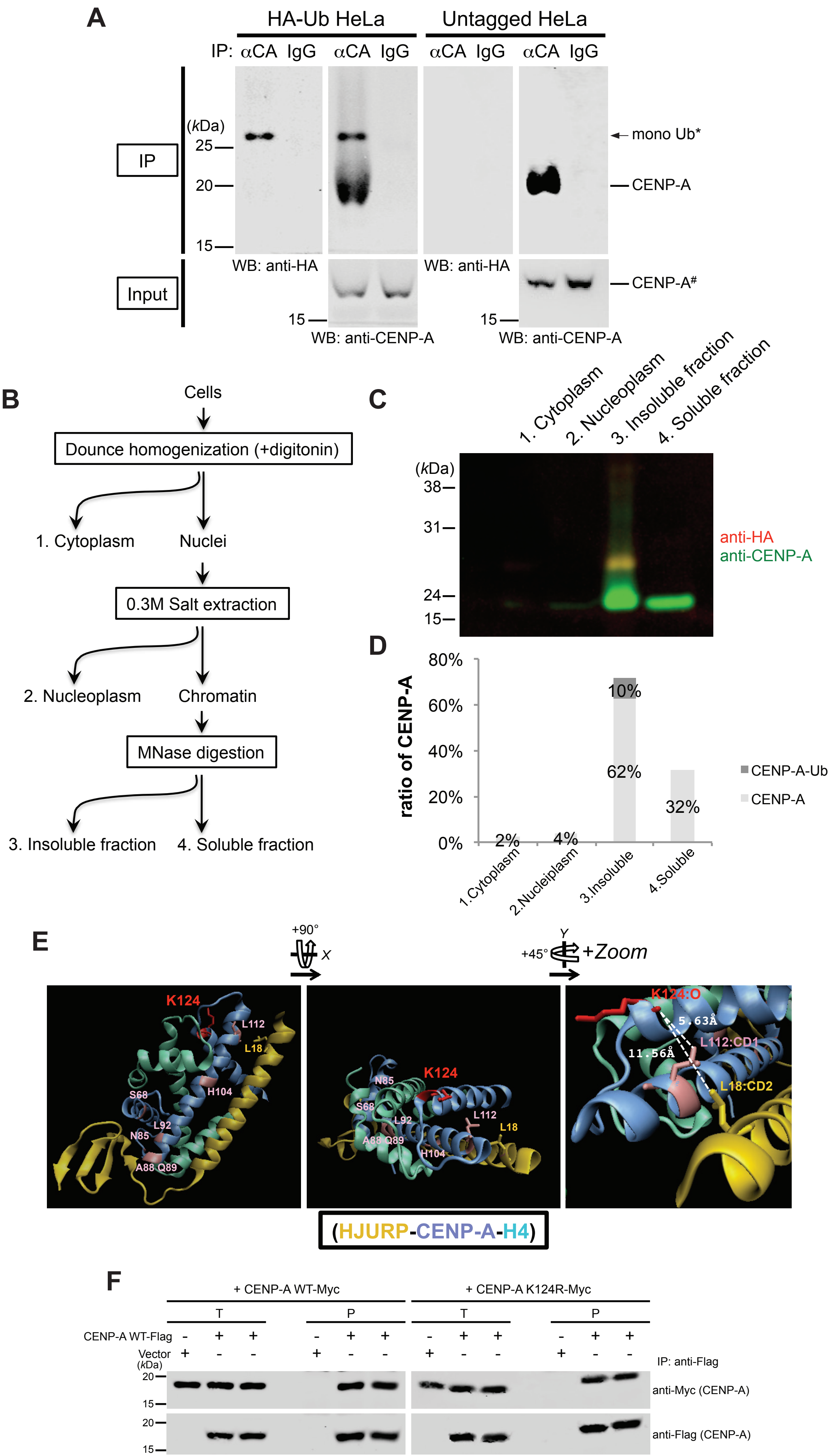
Supplemental Figure 4



Best-fit values	10nM WT	10nM K124R-Ub (K48R)
Bmax (counts)	12711	17249
Kd (nM)	6.04	0.80
Std. Error	**10nM WT**	**10nM K124R-Ub (K48R)**
Bmax (counts)	568	583
Kd (nM)	0.79	0.12

Supplemental Figure 5


A
B
C
D
E
F
G
H
RBX1
CUL4A
Luc
RBX1
CUL4A
Luc
siRNA
72h
48h
Luc siRNA
CUL4A siRNA
RBX1 siRNA
0h
6.5h
8h
15h
23h
32h
DAPI
H3-CATD
CENP-B
Merge
Luc siRNA Prophase
CUL4A siRNA Prophase
RBX1 siRNA Prophase
CUL4A+RBX1 siRNA Prophase
Luc siRNA Metaphase
CUL4A siRNA Metaphase
RBX1 siRNA Metaphase
CUL4A+RBX1 siRNA Metaphase
Luc siRNA Interphase
CUL4A siRNA Interphase
RBX1 siRNA Interphase
CUL4A+RBX1 siRNA Interphase
10μm
Pro/Prometaphase
Metaphase
Interphase
Remaining centromere signals (%)
YFP-H3-CATD
YFP vector
(kDa)
anti-GFP (YFP-H3-CATD)
anti-GAPDH
anti-CUL4A
anti-RBX1
HA-Ub
YFP- vector
IP: anti-GFP
di Ub**
mono Ub*
WB: anti-HA (Ub)
WB: anti-GFP (H3-CATD)
YFP
IP
Input
WB: anti-GAPDH
WB: anti-CUL4A
WB: anti-RBX1
G1/S
S
G2
M
G1
TN16
Asyn
12hr
Chromatin-free extracts
anti-HA (Ub)
anti-Flag
mono-Ub
di-Ub
Fold
TN-16
12 hr
Supplemental Figure 6



Supplemental Figure 7