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Apr 19, 2020

Dear Dr. Vineeta Bajaj,

Enclosed you will find the “revised” manuscript entitled, “Mass spectrometry analysis to identify ubiquitylation of EYFP-tagged CENP-A (EYFP-CENP-A)” by Niikura et al..

Following your guidance, we tracked the changes within the manuscript to identify all of the edits as indicated with left-red line in the file, and put our blue comments in the boxes: **JoVE_YN6_tracked=final.docx.**
We also summarized your comments (black) and our comments (blue) as shown in the file: **Rebuttal Letter_YN1=final.docx**

We believe that we significantly improve the quality of our manuscript and that our methods are of interest to the readership of JoVE.
As we started our discussion in Dr. Kyle Jewhurst’s e-mail after September 26, 2019, I would be grateful if you consider our manuscript for the publication in JoVE.

Thank you very much for your consideration.
Best regards,

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TITLE:

Mass Spectrometry Analysis to Identify Ubiquitylation of EYFP-tagged CENP-A (EYFP-CENP-A)

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KEYWORDS:

centromere, kinetochore, mitosis, CENP-A, post-translational modification, PTM, ubiquitylation, mass spectrometry analysis

SUMMARY:

CENP-A ubiquitylation is an important requirement for CENP-A deposition at the centromere, inherited through dimerization between cell division, and indispensable to cell viability. Here we describe mass spectrometry analysis to identify ubiquitylation of EYFP-tagged CENP-A (EYFP-CENP-A) protein.

ABSTRACT:

Studying the structure and the dynamics of kinetochores and centromeres is important in understanding chromosomal instability (CIN) and cancer progression. How the chromosomal location and function of a centromere (i.e., centromere identity) are determined and participate in accurate chromosome segregation is a fundamental question. CENP-A is proposed to be the non-DNA indicator (epigenetic mark) of centromere identity, and CENP-A ubiquitylation is

required for CENP-A deposition at the centromere, inherited through dimerization between cell division, and indispensable to cell viability.

Here we describe mass spectrometry analysis to identify ubiquitylation of EYFP-CENP-A K124R mutant suggesting that ubiquitylation at a different lysine is induced because of the EYFP tagging in the CENP-A K124R mutant protein. Lysine 306 (K306) ubiquitylation in EYFP-CENP-A K124R was successfully identified, which corresponds to lysine 56 (K56) in CENP-A through mass spectrometry analysis. A caveat is discussed with the use of GFP/EYFP or the tagging of high molecular weight protein as a tool to analyze the function of a protein and current technical limit for the detection of ubiquitylated bands, identification of site-specific ubiquitylation(s), and visualization of ubiquitylation in living cells or a specific single cell during the whole cell cycle.

The method of mass spectrometry analysis presented here can be applied to human CENP-A protein with different tags and other centromere-kinetochore proteins. These combinatory methods consisting of several assays/analyses could be recommended for researchers who are interested in identifying functional roles of ubiquitylation.

INTRODUCTION:

In most eukaryotes, spindle microtubules must attach to a single region of each chromosome, termed as centromere. The kinetochore is a complex of proteins that are located at the centromere. Studying the timing of centromere and kinetochore protein's movements and the structure of kinetochores and centromeres is important for understanding chromosome instability (CIN) and cancer progression. The key questions are how the chromosomal location and function of a centromere (i.e., centromere identity) are determined and how they participate in accurate chromosome segregation. In most species, the presence of a special nucleosome containing a specific histone-like protein called CENP-A defines the centromere identity. Therefore, it is proposed that CENP-A is the non-DNA indicator (epigenetic mark) of centromere identity. It is important to elucidate the mechanism of how CENP-A defines the centromere identity in humans.

The Holliday junction recognition protein (HJURP) is the CENP-A-specific chaperon which deposits CENP-A in centromeric nucleosomes[1-3]. We have previously reported that for CENP-A ubiquitylation on lysine 124 (K124) and centromere localization, CUL4A-RBX1-COPS8 E3 ligase is required[4]. Also, our results showed that the centromere recruitment of newly synthesized CENP-A requires pre-existing ubiquitylated CENP-A[5]. Thus, a model was provided which showed CENP-A ubiquitylation is inherited through dimerization between cell divisions.

In contrast to our findings and those of Yu et al., negative results regarding the CENP-A and its centromeric localization were recently published[6]. The article claimed that CENP-A modifications on lysine 124 (K124) are dispensable for the establishment, maintenance, and long-term function of human centromeres, based on their negative results showing that the mutation of K124R did not affect CENP-A centromere localization neither cell viability[6]. However, there is enough room for debate in their results and conclusions, and we have already described what the problem may be in our previous publication[7]. Attention should be paid that they fused proteins with CENP-A,

which have much larger molecular weights than the size of endogenous CENP-A: e.g., they fused ~30 kDa enhanced yellow fluorescent protein (EYFP) to ~16 kDa CENP-A and analyzed EYFP-CENP-A K124R fusion protein in their RPE-1 CENP-A$^{-/F}$ knockout system. K124 ubiquitin is not expected to bind directly to HJURP based on structural predictions[4], however, addition of mono-ubiquitin is predicted to have an impact on protein conformation. Protein conformation can be changed by the presence of a large fusion protein, and this conformational change may mask the structural changes caused by the loss of ubiquitylation. We suggest that the fusion of large-sized protein induces ubiquitylation at a lysine other than K124 in EYFP-CENP-A K124R mutant and this ubiquitylation at another site inhibits/masks the original K124R single mutant phenotype. Evidence that ubiquitylation occurs at different lysine in the CENP-A K124R mutant protein with a large tag protein (EYFP) was reported in our previous publication[8]. It was found that EYFP tagging induces ubiquitination of another lysine site of EYFP-CENP-A K124R and that EYFP-CENP-A K124R mutant binds to HJURP. As a result, this ubiquitylation at another site inhibits/masks the original K124R single mutant phenotype, and both EYFP-CENP-A WT and K124R mutants showed centromere localization (we used and compared pBabe-EYFP-CENP-A WT and K124R mutant, together with pBabe-EYFP control.). The results demonstrated that Flag-tagged or untagged CENP-A K124R mutants are lethal but can be rescued by a monoubiquitin fusion, suggesting that CENP-A ubiquitylation is indispensable to cell viability.

In recent years, many studies have developed different assays to identify posttranslational modifications (PTMs) of CENP-A protein and other centromere-kinetochore proteins both in vivo and in vitro[9-11]. Analogous to the PTMs of histone proteins that are a major mechanism regulating the function of chromatin, PTMs of centromeric chromatin components are also involved in an essential mechanism to regulate the overall structure and function of centromeres. The majority of CENP-A PTM sites are specific to CENP-A-containing nucleosomes, although a few of them are conserved in histone H3, suggesting that modification of these residues contribute to the centromere-specific function. PTMs of CENP-A including phosphorylation, acetylation, methylation, and ubiquitylation were previously reported[9], suggesting that CENP-A is subjected to a variety of PTMs and their combinatorial arrays on its amino terminus and C-terminus histone-fold domain. The importance of CENP-A modifications in multiple functions was revealed by many groups including ours. These functions involve CENP-A deposition at centromeres, protein stability, and recruitment of the CCAN (constitutive centromere-associated network)[9]. However, limited studies and findings of CENP-A PTMs are preformed where comparisons are made with one of canonical histones that directly or indirectly regulate their function. Technical reports focusing on the methodology to identify these CENP-A PTMs are also limited.

Because CENP-A ubiquitylation is required for CENP-A deposition at the centromere[12], inherited through dimerization between cell division[5], and indispensable to cell viability[8], the method to identify CENP-A ubiquitylation would be essential in future to study the functional activity, positioning, and structure of the centromere. Therefore, here we describe mass spectrometry analysis to identify ubiquitylation of EYFP-CENP-A K124R mutant suggesting that the EYFP tagging induces ubiquitylation at a different lysine in the CENP-A K124R mutant protein[8]. Protocols of other control assays and analyses (immunofluorescence analysis, colony outgrowth assay, and in vivo ubiquitylation assay) are also presented to discuss the outcome of major mass spectrometry

analysis properly.

PROTOCOL:

1. Cell culture and retrovirus transfection of pBabe-EYFP-CENP-A constructs

NOTE: EYFP-CENP-A is expressed from pBabe-EYFP-CENP-A at a similar protein level to endogenous CENP-A. Total cellular CENP-A protein is replaced with this EYFP-CENP-A after the disruption of the CENP-$A^{-/F}$ allele by Cre recombinase as in RPE-1 CENP-A-/- cells[6].

1.1. Preparation of the supernatant containing retrovirus using 293T packaging cells.

1.1.1. Day 0: Spread 293T packaging cells on the 6-well culture plate (1.0 x 10^6 cells/well). Culture cells in high-glucose DMEM with 10% FBS and 1% penicillin-streptomycin. Incubate the cells at 37 °C in an atmosphere of 5% CO_2 for 24 h.

NOTE: For optimal results, empirically determine the cell density to use in seeding.

1.1.2. Day 1: Prepare transfection reaction around 23 h after spreading (24 h point is 0 h point of the transfection).Transfect expression plasmid of each pBabe-EYFP (B3182), pBabe-EYFP-CENP-A WT (B3161), and pBabe-EYFP-CENP-A K124R (B3164) (see **Table 1**).

1.1.3. Choose one combination of helper/packaging plasmids listed in **Table 2** and add them to tubes containing one of the plasmids listed above. There are mostly these 3 combinations for helper/packaging plasmids (**Table 2**). Any combination worked in these experiments and showed similar transfection efficiency.

1.1.4. Prepare 50 µL of reduced serum medium and mix 2.0 µg of each pBabe-EYFP (B3182), pBabe-EYFP-CENP-A WT (B3161), and pBabe-EYFP-CENP-A K124R (B3164) (see **Table 1**) adding one combination of helper/packaging plasmids listed in **Table 2** (see below). Incubate this mixture at room temperature for 5 min. This mixture is solution A.

1.1.5. Prepare another 50 µL of reduced serum medium and mix 1.5 µL of transfection reagents I and II, respectively (**Table of Materials**). Incubate this mixture at room temperature for 5 min. This mixture is solution B.

NOTE: Optionally, add only 6.0 µL of transfection reagent III (polyethyleneimine [PEI]; 1.0 mg/mL) in solution B or add 6.0 µL of transfection reagent III in addition to transfection reagents I and II in solution B (**Table of Materials**).

1.1.6. Mix solutions A and B together, and incubate at room temperature for 15 min.

1.1.7. After washing the cultured cells once with PBS, add the mixture of solutions A and B (i.e., DNA-lipid complex) directly to each well of the 6-well culture plate that has 500 µL reduced serum

medium. The final concentration of the plasmid is 3.3 μg/mL.

1.1.8. After incubating the cells at 37 °C in an atmosphere of 5% CO_2 for 4.5 h, change the medium to high-glucose DMEM with 10% FBS and 1% penicillin-streptomycin. Put 2 mL/well of culture medium after the medium change in the 6-well culture plate.

1.1.9. Culture the cells at 37 °C in an atmosphere of 5% CO_2 for 48 h after transfection. Perform retrovirus infection on Day 3 using the supernatant containing retrovirus.

1.2. Retrovirus infection of CENP-$A^{-/F}$ RPE-1 target cells.

1.2.1. Day 2: A day before infection, spread CENP-$A^{-/F}$ RPE-1 target cells to transfect in the 6-well culture well (2.25 x 10^5 cells/well). Culture cells in DMEM: F12 medium (**Table of Materials**) with 10% FBS and 1% penicillin-streptomycin.

NOTE: Spread cells for colony outgrowth assays, immunofluorescence, and western blot analysis as control. For optimal results, empirically determine the cell density to use in seeding.

1.2.2. Day 3 (infection day of the virus): At 48 h after transfection of 293T packaging cells, collect the supernatant containing virus and filter through 0.45 μm filter (do not use a 0.2 μm filter that will shear virus envelope). It is recommended to use polyethersulfone (PES) filters.

1.2.3. Infect CENP-$A^{-/F}$ RPE-1 cells with the virus. For one well of the 6-well culture plate, add 1 mL fresh media, 1 mL virus supernatant and polybrene to a final concentration of 8 μg/mL. Incubate CENP-$A^{-/F}$ RPE-1 cells at 37 ˚C in an atmosphere of 5% CO_2 for 4 days until Day 7 after transfection.

NOTE: The incubation period for CENP-$A^{-/F}$ RPE-1 cell growth must be determined empirically by following analyses for optimal results.

2. Immunofluorescence analysis of cells containing pBabe-EYFP-CENP-A

2.1. Day 7: 4 days after retrovirus infection of pBabe-EYFP-CENP-A constructs remove the culture medium by aspiration. Rinse cells once with TBS (25 mM Tris-HCl, pH 7.5; 125 mM NaCl). Apply TBS to the side of the culture wells to avoid disturbing the surface of cells.

NOTE: The optimal time point for cell fixation must be determined empirically. Immunofluorescence signals of both EYFP-CENP-A WT and K124R are detectable at the centromere at least 4 days after retrovirus infection of pBabe-EYFP-CENP-A constructs even without Cre infection (data not shown, **Figures 1C-E** for the data with Cre infection).

2.2. Perform methanol cell fixation and immunofluorescence staining as described previously[13].

2.3. As primary antibodies use an anti-GFP antibody (1:1,000 dilution) and an anti–CENP-B

antibody (dilution ratio of 1:200) for a centromere location marker in TBS containing 4% goat serum (see **Table of Materials** for antibodies used).

2.4. Remove excess mounting medium with a paper towel and seal the edges of the coverslip with nail polish at the last step.

2.5. Refer to the previously described method[13] for immunofluorescence image observation, acquisition, quantification, and analysis of remaining signals of EYFP-CENP-A at the centromere.

2.6. Perform image acquisition, processing including deconvolution, quantification, and analysis using Softwares A or Softwares B1 and B2 (see **Table of Materials**). Optionally use Softwares C1-C3 (see **Table of Materials**) for a confocal laser scanning microscope.

NOTE: See 2.6.1 in Supplemental coding files for all commands used in Software A. See 2.6.2 in Supplemental coding files for all commands used in Softwares B1 and B2.

3. **Colony outgrowth assays using pBabe-EYFP-CENP-A after retro-Cre virus infection**

NOTE: The reason for performing this assay is to compare the cell viability between EYFP-CENP-A WT and K124R mutant after the disruption of the CENP-$A^{-/F}$ allele by Cre recombinase (after the replacement of total cellular CENP-A protein).

3.1. Retrovirus transfection of pBabe-EYFP-CENP-A constructs.

3.1.1. Perform retrovirus transfection of CENP-$A^{-/F}$ RPE-1 cells as described in section 1. Culture cells in DMEM: F12 medium with 10% FBS and 1% penicillin-streptomycin for 72 h after the virus infection.

3.1.2. Three days after retrovirus infection of pBabe-EYFP-CENP-A constructs (on Day 6), add blasticidin S (10 μg/mL) in wells containing transfected cells to be used for colony outgrowth assay and control experiments. Cells are grown at least 14 days after virus infection in the presence of blasticidin S. Change the medium containing blasticidin S every 5 days.

NOTE: If cells reach about 80% confluence before seeding for the colony assay (3.2.7. and 3.2.8), passage the cells at 1:2 and 1:5 ratio by trypsinization and plating in a 6 well culture plate.

3.1.4. Collect cells for western blot on Day 7 to confirm the protein expression of pBabe-EYFP-CENP-A constructs without Cre infection. Perform western blot analysis as described in section 4. The results are shown in **Figure 1B** (lanes 1-4).

3.1.5. For colony outgrowth assay with Cre virus infection, keep the cells growing for 14 days after the virus infection in the presence of blasticidin S (i.e., grow cells in blasticidin S containing medium until Day 17 for for the results presented here).

3.2. Retro-Cre virus infection of pBabe-puro-Cre

NOTE: Day 0 in step 3.2.1 corresponds to Day 13 of in section 3.1.

3.2.1. Day 0 to Day 3: Perform retrovirus transfection of CENP-$A^{-/F}$ RPE-1 cells using pBabe-puro-Cre (B3027) as expression plasmid (see section 1 for details). Ensure that blasticidin S (10 µg/mL) is added in the culture medium.

3.2.2. Day 4: Trypsinize cells to detach it from the plate. Plate 500 or 5,000 cells in triplicate in the 6-well culture plate. Culture cells in DMEM: F12 medium with 10% FBS and 1% penicillin-streptomycin.

3.2.3. Day 5: Add blasticidin S (10 µg/mL) to the culture medium. For 5,000 or 500 cells' plating, select cells with blasticidin S (10 µg/ml) 3-24 days (until Day 14 in [3.2.7]) or 3-28 days (until Day 18 in [3.2.8]) after virus infection of constructs (pBabe-EYFP-CENP-A constructs).

NOTE: In this colony outgrowth assay, the transfection of pBabe-puro-Cre is added along with the transfection of of pBabe-EYFP-CENP-A. The transfection of pBabe-EYFP-CENP-A is performed in 3.1. The transfection of pBabe-puro-Cre is performed in 3.2.1.

3.2.4. Day 10 (7 days after retro-Cre virus infection): Collect cells for western blotting analysis to confirm protein depletion of endogenous CENP-A after retro-Cre virus infection and/or protein expression of pBabe-EYFP-CENP-A constructs. on the same day 10.

3.2.5. Perform western blotting as described in section 4. Results are shown in **Figure 1B** (lanes 5-7).

3.2.6. Perform immunofluorescence analysis (section 2 above) to confirm that both EYFP-CENP-A WT and K124R localized to the centromere 7 days after inactivation of the remaining endogenous CENP-A allele. The results are shown in **Figures 1C-1E**.

3.2.7. Day 14: Perform the colony outgrowth assay with the plate seeded with 5,000 cells. Fix cells for 10 min in methanol and stain for 10 min in a crystal violet solution (2.3% crystal violet, 0.1% ammonium oxalate, 20% ethanol (see **Figure 2B**). Count the number of colonies using the OpenCFU software (see **Figure 2C**).

3.2.8. Day 18: Use the plate seeded with 500 cells. fix and stain cells as described in step 3.2.7 (see **Figure 2B**). Count the number of colonies (see **Figure 2C**).

4. Western blot analysis using pBabe-EYFP-CENP-A

NOTE: Refer to the previously described method [13] for Western blot analysis using antibodies indicated in **Figures 1B** and **2A** and **Table of Materials** for EYFP-CENP-A proteins.

4.1. Isolate proteins by lysing the cells grown with different virus infection. Then use 1 µg of protein to run an SDS PAGE. Transfer the proteins to a PVDF membrane as described previously[13].

4.2. Wash the membrane 3x after incubations with primary-secondary antibodies. Detect and analyze the protein bands on the membrane with the infrared imaging system and/or the chemiluminescence imager for immunoblot detection. These results are shown in **Figures 1B** and **Figure 2A**.

4.2.1. For the infrared imaging system to detect and analyze the protein bands, see step 4.2.1 in **Supplemental coding files**.

4.2.2. Use the chemiluminescence imager to detect and analyze the protein bands, see step 4.2.2 in **Supplemental coding files**. For this system, use an ultra-sensitive enhanced chemiluminescent (ECL) substrate.

4.2.3. Optionally, use western blot stripping buffer to strip out pre-incubated antibodies from the PVDF membrane and reblot it with different antibodies for the next turn of western analysis. Empirically determine the optimized incubation time and temperature to use.

5. Cell Culture, transfection, and in vivo ubiquitylation assays using pQCXIP-EYFP-CENP-A

NOTE: The protein level of EYFP-CENP-A expressed from pQCXIP-vector is ~ 10x higher than the endogenous CENP-A protein level. The usage of this vector facilitates immunoprecipitation of a higher amount of the EYFP-CENP-A proteins, observation of the ubiquitylation bands of EYFP-CENP-A, and identification of the ubiquitylation of EYFP-tagged CENP-A (EYFP-CENP-A) protein through mass spectrometry analysis.

5.1. Transfection for in vivo ubiquitylation assays using pQCXIP-EYFP-CENP-A.

5.1.1. Seed 36.2 x 10^5 CENP-$A^{-/F}$ RPE-1 cells in a 10 cm tissue culture dish. Culture cells in high-glucose DMEM with 10% FBS and 1% penicillin-streptomycin.

NOTE: For optimal results, empirically determine the cell density to be used in seeding. Prepare at least 2 dishes for one immunoprecipitation (IP) sample to obtain a minimum of 1 mg total protein.

5.1.2. Incubate the cells at 37 °C in an atmosphere of 5% CO_2 for 18 h.

5.1.3. At 17 h after seeding, prepare the transfection regent.

5.1.4. Make solution A by mixing 6.7 µg plasmid of each pQCXIP-EYFP (B3252), pQCXIP-EYFP-CENP-A WT (B3254), pQCXIP-EYFP-CENP-A K124R (B3256) (**Table 1**) in 335 µL of reduced serum medium, and incubate at room temperature for 5 min. Add 6.7 µg plasmid of pCGN-HA-Ubiquitin (B2806) to all samples.

NOTE: All the vectors are listed in **Table 1**.

5.1.5. Make solution B by mixing 10.1 µL of transfection reagents I and II, respectively in 335 µL reduced serum medium, and incubate at room temperature for 5 min.

NOTE: An optional step is to add only 40.2 µL transfection reagent III (polyethyleneimine [PEI]; 1.0 mg/mL) in solution B or add 40.2 µL transfection reagent III in addition to transfection reagents I and II in solution B (**Table of Materials**).

5.1.6. Mix solutions A and B together, and incubate at room temperature for 15 min.

5.1.7. After washing the cultured cells once with PBS, add the mixture of solutions A and B (i.e., DNA-lipid complex) directly to each of the individual 10 cm tissue culture dish that has 3.35 mL µL reduced serum medium.

NOTE: The final concentration is 1.67 µg/mL of plasmid.

5.1.8. Incubate the cells at 37 °C incubator with 5% CO_2 for 4.5 h. After 4.5 h, change the medium to high-glucose DMEM with 10% FBS and 1% penicillin-streptomycin.

5.1.9. Culture the cells at 37 °C with 5% CO_2 for 48 h after transfection. Collect cells for cell lysates preparation.

5.2. Preparation of protein A beads bound with anti-GFP antibody.

5.2.1. Take 25 µL (50% v/v) of protein A beads for one reaction of immunoprecipitation (IP). Wash with buffer A1 (20 mM Tris-HCl, pH 7.4; 50 mM NaCl; 0.5% Nonidet P-40; 0.5% deoxycholate; 0.5% SDS; 1 mM EDTA; complete EDTA-free protease inhibitor reagent) at least 3x to remove EtOH, and make 50% solution with buffer A1.

5.2.2. Add 2.0 µL of anti-GFP antibody (Anti#76: Homemade antibody) to the beads prepared above and add 10x volume of buffer A1 comparing with net beads volume.

5.2.3. Perform end-to-end rotation at 4 °C for 4-18 h. The optimal time length for end-to-end rotation must be determined empirically based on the efficiency of the immunoprecipitation.

5.2.4. Centrifuge the beads at 100 x *g* for 1 min and remove the unbound supernatant. Add buffer A1 to re-make a 50% (v/v) solution of the beads. Use 25 µL (50% v/v) of this solution for one reaction of IP.

5.3. Immunoprecipitation (IP) using protein A sepharose beads bound with anti-GFP antibody.

5.3.1. Lyse cells obtained in step 5.1.9 in buffer A1 by sonication and freeze-thaw process.

5.3.2. Measure protein concentrations and normalize protein amounts among different IP samples. Remove 5% of the sample from each tube to run as 5% Input sample in SDS page.

NOTE: The 5% Input sample can be frozen in liquid nitrogen and stocked at -80 °C if it is not loaded within a day.

5.3.3. Mix the rest of 95% lysate with 25 µL (50% v/v) of protein A beads bound to anti-GFP antibody that was prepared in step 5.2. Perform end-to-end rotation at 4 °C for 4-18 h.

NOTE: The optimal time length for end-to-end rotation must be determined empirically.

5.3.4. Centrifuge protein A beads with the protein bound to it (i.e, immunoprecipitates) with 100 x *g* for 1 min, and remove the supernatant. Wash the immunoprecipitates with buffer A1 by centrifuging at 100 x *g* for 1 min. Perform this step 4x.

5.3.5. Mix the 5% Input and the rest of 95% immunoprecipitates with 2x and 4x SDS-PAGE loading buffer[14], respectively. Boil these two samples for 5 min and then load them on a 10.0% denaturing SDS-polyacrylamide gel for electrophoresis in different lanes. Use bigger SDS-PAGE gel (e.g., 17 cm x 15 cm) for electrophoresis. If samples are run in smaller gel, it may not be possible to observe clear/sharp ubiquitylation bands.

5.3.6. Perform western blot analysis as described in section 4 using the antibodies indicated in the previous report [8]. The result is shown in **Figure 2A**.

5.3.7. Use western blot stripping buffer to strip out the pre-incubated antibodies from the PVDF membrane and reblot it with different antibodies for the next round of western blot analysis.

6. Mass spectrometry to identify the ubiquitylation site of the EYFP-CENP-A K124R mutant

6.1. Transfection for mass spectrometry analysis using pQCXIP-EYFP-CENP-A.

6.1.1. Seed CENP-A$^{-/F}$ RPE-1 cells in a 10 cm tissue culture dish. Check that cell density is 36.2 x 10^5 cells per dish. Culture cells in high-glucose DMEM with 10% FBS and 1% penicillin-streptomycin. Prepare at least 10 dishes for one immunoprecipitation (IP) sample, to obtain at least 20 mg total protein (see 5.3).

NOTE: For optimal results, empirically determine the cell density to use in seeding.

6.1.2. Incubate the cells at 37 °C in an atmosphere of 5% CO_2 for 18 h. Prepare the transfection regent ~ 23 h after spreading (24 h point is 0 h point of the transfection).

6.1.3. At 17 h after seeding, prepare the transfection reagents and transfect cells as (4.1.3). Transfected cells has pCGN-HA-Ubiquitin (B2806) and pQCXIP-EYFP-CENP-A K124R (B3256).

6.1.4. Incubate the cells at 37 °C in an atmosphere of 5% CO_2 for 48 h after transfection. Collect cells for cell lysates.

6.1.5. Lyse cells in buffer A1 and perform immunoprecipitation as (5.2) and (5.3). Run these two of immunoprecipitation lysates as following (6.1.6) and (6.1.7).

NOTE: In (6.1.6), run the sample in standard Tris-glycine gels. In (6.1.7), run the sample in the commercially available 4%-12% Bis-Tris protein gels. See also Discussion.

6.1.6. Keep one sample for 10% of total immunoprecipitants to confirm EYFP-CENP-A ubiquitylation and to precisely determine the position of ubiquitinated EYFP-CENP-A as described in section 5. Run this sample in standard Tris-glycine gels. SDS-PAGE and western blot of anti-CENP-A and anti-ubiquitin were performed as (5.3).

6.1.7. Keep another sample for 90% of total immunoprecipitants for mass spectrometry analysis. Run this sample in the commercially available 4%-12% Bis-Tris protein gels. Perform Coomassie blue staining using Coomassie blue solution. Excise and cut out the gel region of 50-70 kDa. Use this gel region for mass spectrometry analysis.

6.2. Mass spectrometry analysis using in-gel digestion.

6.2.1. Dice each gel slice of interest into small pieces (1 mm^2) and place it into 0.5 mL of protein low binding tubes.

6.2.2. Wash with 100 µL 50% (v/v) acetonitrile in 25 mM NH_4HCO_3, vortex 10-15 min, spin down, discard the supernatant, repeat 3x.

6.2.3. Concentrate the sample using benchtop vacuum concentrator for 30 min to dry the gel pieces.

6.2.4. Add 10 µL of 10 ng/µL sequencing grade trypsin and let the gel pieces to rehydrate for 5 min.

6.2.5. Add 25 mM NH_4HCO_3 just enough to cover the gel pieces, digest at 37 °C overnight.

6.2.6. Transfer the digested supernatant into a clean 0.65 mL siliconized tube. Add 50% (v/v) acetonitrile/5% (v/v) formic acid (30 µL or enough to cover), vortex 10 min, spin and transfer into the same extraction tube. Repeat 3x.

6.2.7. Add 10 µL acetonitrile to the gel pieces, vortex 5 min, and spin down. Transfer the supernatant to the same tube.

6.2.8. Concentrate the samples using benchtop vacuum concentrator to 2 µL, add 8 µL 3% (v/v)

acetonitrile /2% (v/v) formic acid to the sample, vortex for 15 min, and spin down at 16,000 *x g* for 30 min. Samples are ready for mass spectrometry analysis.

6.2.9. Perform MS data acquisition with LC-MS/MS using a liquid chromatography system (**Table of Materials**) coupled with a mass spectrometry instrument (**Table of Materials**).

6.2.10. Inject 8 µL of reconstituted sample onto a reverse phase liquid chromatography (RPLC) column.

6.2.11. Separate the peptides with a 2-80% gradient of solvent B in 60 min. Ensure that the gradient consists of an increasing percentage of solvent B from 2% to 22% in 40 min, 22% to 35% solvent B in 12 min, then climbing to 80% solvent B in 4 min, and finally holding at 80% solvent B for the last 4 min. Set the flow rate constant at 300 nL/min.

NOTE: Solvent A contains 0.1% formic acid and 2% acetonitrile, solvent B contains 0.1% formic acid and 98% acetonitrile. All concentrations are shown as volume/volume.

6.2.12. Collect mass spectrometry data using data-dependent acquisition mode. Briefly, collect MS spectra in 350–1500 m/z for 250 ms. Select the Top 50 intense precursors with charge 2–5 for further fragmentation. Collect MS/MS spectra in 100–2000 m/z for 100 ms, Exclude precursor ions from reselection for 15 s.

6.2.13. For database searching, open the commercial software (see **Table of Materials**) to analyze mass spectrometry data on the desktop.

6.2.14. To make a new search, click the "**LC**" button on the top menu. Then click the "**Add**" button to upload the original MS raw data files.

6.2.15. Select "**Human Protein ID**" in the "**Paragon Method**" as the database searching method. Search the original MS raw data files against the UniProt Homo Sapiens database (containing 160,566 sequences, http://www.uniprot.org/proteomes/UP000005640).

6.2.16. Set searching parameters as the following: select **trypsin** as the digestion enzyme, allow up to 3 missing cleavages, 4 modifications and 2-5 charges per peptide. Set **mass error** up to 20 ppm for the first search, and 0.02 Da for fragmented ions. Specify **false discovery rate** (FDR) thresholds for protein, peptide, and modification sites less than 1%. Set all the other parameters in the software to default values (see **Figure 3** for mass spectrometry analysis).

6.2.17. Click the "**Save as**" button on the right of the top menu, select a folder for storing the searching results, enter the search name and click the "**Save**" button.

6.2.18. Click the "**Process**" button on the right of the top menu to start the searching. After the search ends, data with the entered search name will be automatically stored in the folder selected. The data can be easily opened by Software F.

6.2.19. To obtain MS/MS spectra of any specific peptide, double click the search results to open it by Software F. First, click the protein in the protein list on the top of the menu, then click the peptide on the middle of the menu. The MS/MS of this peptide is shown on the bottom of the menu.

6.2.20. To export and save MS/MS spectra, right-click on the MS/MS spectra on the bottom of the menu, select copy and then paste to a suitable file such as PPT or doc. format.

REPRESENTATIVE RESULTS:

EYFP-CENP-A K124 mutant shows ubiquitylation, interaction with HJURP, and no defects in centromere localization neither cell lethality. Here the system reported by Fachinetti et al. (2017)[6] was re-constituted: in diploid human (RPE-1) cells carrying one disrupted and one ''floxed'' *CENP-A* allele (*CENP-A*$^{-/F}$), EYFP-CENP-A was expressed from the pBabe-EYFP retrovirus vector. In this system, the expression of endogenous CENP-A from the *CENP-A*$^{-/F}$ allele could be disrupted by Cre recombinase[6]. Gene constructs of EYFP- CENP-A WT or K124R mutant (**Figure 1A**), which rescues the loss of endogenous CENP-A, were stably expressed when retroviral integration was performed. The remaining expression of endogenous CENP-A from the *CENP-A*$^{-/F}$ allele was then disrupted by Cre recombinase. The expression of endogenous CENP-A was not detected 7 days after the induction of Cre recombinase (**Figure 1B**, lanes 5-7). Both EYFP-CENP-A WT and K124R proteins expression was found to be at a similar level to the initial endogenous CENP-A protein level (**Figure 1B**, lanes 3, 4, 6, and 7). Both EYFP-CENP-A WT and K124R mutants showed centromere localization at 7 days after the disruption of the remaining expression of endogenous CENP-A from the *CENP-A*$^{-/F}$ allele (**Figures 1C-1E**). Both EYFP-CENP-A WT and the K124R mutant showed ubiquitylation and interaction with HJURP unlike the case of Flag-tagged or untagged CENP-A WT and the K124R mutant (**Figure 2A**; data not shown for Flag-tagged or untagged CENP-A)[8]. The cell viability was also addressed by performing the colony outgrowth assay 14 days after the disruption of the remaining endogenous *CENP-A* allele (**Figure 2B**). Both EYFP-CENP-A WT and K124R mutants showed a similar number of ''rescued'' colonies 14 days after the disruption of the remaining endogenous *CENP-A* allele (**Figures 2B** and **2C**). Thus, our results are in line with those reported by Fachinetti et al.[6].

Ubiquitylation at lysine 306 (K306) in EYFP-CENP-A K124R was revealed by mass spectrometry analysis. It was found that both EYFP-CENP-A WT and the K124R mutant shows ubiquitylation and interaction with HJURP unlike the case of Flag-tagged or untagged CENP-A WT and the K124R mutant (**Figure 2A**; data not shown for Flag-tagged or untagged CENP-A)[8]. These results suggest that the fusion of EYFP protein induces ubiquitylation at a lysine other than K124 in EYFP-CENP-A K124R mutant, and this ubiquitylation at another site promote the interaction of EYFP-CENP-A K124R with HJURP. Ubiquitylation at lysine 306 (K306) in EYFP-CENP-A K124R was revealed in CENP-A$^{-/F}$ cells by IP-mass spectrometry analysis (**Figures 3A** and **3B**). The lysine 306 (K306) in EYFP-CENP-A K124R corresponds to lysine 56 (K56) in CENP-A. Taken together, our results suggest that the fusion of large-sized protein (e.g., EYFP-tagging) induces ubiquitylation at a lysine other than K124 in CENP-A, and this ubiquitylation at another site inhibits/masks the original K124R

single mutant phenotype.

FIGURE AND TABLE LEGENDS:

Figure 1: EYFP-CENP-A K124 mutant localizes at centromeres. (**A**) Representations of the different CENP-A protein constructs tagged with EYFP (enhanced yellow fluorescent protein) at the N terminus. Red letters mark the position of K124R amino acid substitution in the indicated construct (left). The results of the assays performed in this study are shown (right). (**B**) Western blot analysis did not show the presence of detectable endogenous CENP-A. Immunoblots analysis to check for the presence of expression of the indicated rescue constructs (ca. 45 kDa) before and after Cre infection. The protein expression of pBabe-EYFP-constructs was confirmed before Cre infection (lanes 1-4), as well as after Cre infection (lanes 5-6). The absence of the endogenous CENP-A protein (ca. 15 kDa) was confirmed in the CENP-$A^{-/-}$ cell lines collected at 7 days after Cre infection (lanes 5-6). GAPDH protein was used as a loading control. (**C**) EYFP-CENP-A K124 mutant localizes at centromeres. CENP-$A^{-/F}$ RPE-1 cells were cotransfected with indicated constructs, cultured 7 days after retro-Cre virus infection, and immunostained. Visualization of DAPI (blue), EYFP (green), and endogenous CENP-B (red), which served as a centromere location control. Scale bar, 10 μm. (**D**) The localization patterns shown in (C) summarized as histograms. More than 200 interphase cells with EYFP-positive signals were counted per experiment ($n \geq 3$ experiments), and the mean percentages (±SD) are shown. ''Others (Non-centromere)'' depicts mostly damaged cells, dead cells, or cells with nucleolar localization in interphase, which were observed because of transfection or other treatments. No significant (n.s.) difference was observed in K124R comparing to WT (Student's t-test). (**E**) EYFP-derived signals at centromeres shown in (C) were quantified. Signals were normalized to those of WT, and the mean percentages (±SEM) are shown.

Figure 2: EYFP-CENP-A K124 mutant is ubiquitylated and interacts with HJURP, and cell viability was not affected by K124R mutation of EYFP-CENP-A. (**A**) The EYFP-CENP-A K124 mutant is ubiquitylated and interacts with HJURP. In vivo ubiquitylation assay. CENP-$A^{-/F}$ RPE-1 cells were transfected with the indicated constructs. Proteins in 5% of the total cell lysates (Input) and immunoprecipitates (IP) using anti-GFP rabbit polyclonal antibody were detected by western blot analysis using the indicated antibodies. Putative di-Ub-EYFP-CENP-A (**) and putative mono-Ub-EYFP-CENP-A (*) are indicated. This figure has been modified from Niikura et al.[8]. (**B**) Representative images from the colony outgrowth assay as shown in the scheme (top) of two different conditions ([1] and [2]) for the indicated transfectants of EYFP-CENP-A. (**C**) Histograms summarizing colony survival of the experiments in (B). The mean percentages (±SEM) of more than 3 independent experiments ($n \geq 3$) are normalized with the percentage of surviving colonies in EYFP-CENP-A WT (EYFP-WT). ****$p < 0.0001$ and **$p < 0.01$ compared with EYFP control (Student's t-test). No significant (n.s.) difference was observed in K124R comparing to WT (Student's t-test).

Figure 3: Fragments of ubiquitylated EYFP-CENP-A K124R peptides were detected in by mass spectrometry analysis. (**A**) Evidence of ubiquitylation at lysine 306 (K306) of EYFP-CENP-A K124R in RPE-1 CENP-$A^{-/F}$ cells. The lysine 306 (K306) of EYFP-CENP-A K124R corresponds to lysine 56

(K56) in CENP-A. The LQK^{LRGG}STHLLIR peptide (coverage 95.9%, confidence 87.8%) identified by collision-induced dissociation analysis is displayed. The m/z (Da) values of the b (green) and y (red) ions in the spectra of (A) detected during fragmentation are highlighted with green in the table of (B). The ubiquitylation of K306 is confirmed by the LRGG motif (incomplete cleavage) of the b-3 ion (m/z 753.4730) and y-8 ion (m/z 1350.8328). (**B**) The table highlights the m/z (Da) values of the b (green) and y (red) ions in the spectra of (A). LQK$^{[Umc]}$STHLLIR in the table of (B) indicates the LQK^{LRGG}STHLLIR peptide shown in (A). These figures have been modified from Niikura et al.[8].

Table 1. Plasmid vectors used in this study.

Table 2. Combinations of helper/packaging plasmid vectors used in (1.1.3): Retrovirus transfection of pBabe-EYFP-CENP-A constructs.

DISCUSSION:

Here we described methods of mass spectrometry analysis to identify ubiquitylation of EYFP-CENP-A K124R mutant suggesting that the EYFP tagging induces ubiquitylation at a different lysine in the CENP-A K124R mutant protein[8]. In our results, we successfully identified ubiquitylation on lysine 306 (K306) in EYFP-CENP-A K124R, that is corresponding to lysine 56 (K56) in CENP-A through mass spectrometry analysis. The mass spectrometry analysis described here is a mimic method as we previously identify the lysine 124 (K124) ubiquitylation site of CENP-A WT-Flag[12]. Therefore, this method can be applied to human CENP-A protein with different tags and other centromere-kinetochore proteins. The mass spectrometry analysis based on LC-MS/MS is commonly accepted to identify potential posttranslational modifications (PTMs) of a wide spectrum of proteins. Our combinatory methods consisting of several assays/analyses (i.e., in vivo ubiquitylation assay, colony outgrowth assay, and mass spectrometry analysis) could be recommended for researchers who are interested in identifying functional roles of ubiquitylation(s) of their target protein(s).

However, this protocol does not cover the detection of ubiquitylated bands and/or identification of site-specific ubiquitylation(s) of these proteins in living cells or a specific single cell during the whole cell cycle. These years optogenetic approaches are developed dramatically and giving a high impact on quantitative studies of cell-signaling systems. Optogenetics has originally provided approaches that precisely activate or inhibit individual neurons using single-component, microbial opsin-based systems. Currently, protein activity with unprecedented spatiotemporal precision can be controlled by exploiting natural genetically encoded photoreceptors, and various genetically encoded tools allow light-control of many biological processes including protein phosphorylation[15]. Therefore, optogenetics is a promising system to investigate spatiotemporal protein kinase signaling at the cellular and the entire organism levels. The number of light-controlled protein kinases is rapidly expanding, although the current number is still limited. However, the development of light-controlled protein ubiquitylation is delayed, and high molecular weight tagging of photoreceptors may disrupt the ubiquitylation of both WT and mutant proteins and functionally alter the native protein function as aforestated. Therefore,

659 the development of lower molecular weight tagging or probing technique is urgently required to
660 visualize ubiquitylation and/or to investigate spatiotemporal protein ubiquitylation signaling at
661 the living cellular and the entire organism levels.
662
663 In the present study, EYFP vector control is essential for control assays and analyses
664 (immunofluorescence analysis, colony outgrowth assay, and in vivo ubiquitylation assay) to
665 discuss the outcome of major mass spectrometry analysis properly. Non-specific interaction with
666 EYFP-protein is often observed in the immunoprecipitation experiment, thus EYFP vector control
667 is indispensable to evaluate the true interaction of EYFP-fused protein(s). Our mass spectrometry
668 analysis using EYFP-CENP-A K124R expressed in the RPE-1 CENP-$A^{-/F}$ cells did not show
669 ubiquitylation on lysine sites in the EYFP polypeptide sequence. However, in other unknown
670 conditions, it could be expected that the lysine site in the EYFP polypeptide sequence is
671 ubiquitylated in EYFP- and/or other tagged fusion protein of high molecular weight.
672
673 For colony outgrowth assays, it is recommended to collect cells for western blot analysis to
674 confirm protein depletion of endogenous CENP-A after retro-Cre virus infection and protein
675 expression of pBabe-EYFP-CENP-A constructs. In this way, we can judge if the colony outgrowth
676 phenotype is truly due to the sole expression of exogenous CENP-A WT or mutants. For any
677 western blot analysis, if stripping is performed to reblot the membrane with different antibodies
678 for the next turn of western blot analysis, one should use the same quantitative detection system
679 as a previous turn's blot for the next turn's blot with different antibodies. One should make sure
680 that the bands in the previous turn's blot are undetectable at the aimed region of the membrane
681 in the next turn's blot with different antibodies. Or use the same quantitative detection system
682 as previous turn's blot and check if the incubation with the mere secondary antibody used in the
683 previous turn's blot does not lead to the detection of protein bands before starting the next turn's
684 blot with different antibodies. For in vivo ubiquitylation assay, it is important to run bigger SDS-
685 PAGE gel using the apparatus to run bigger SDS-PAGE gel (Gel electrophoresis apparatus I or II).
686 Empirically, bigger SDS-PAGE gel would separate protein bands clearly and enhance the
687 sensitivity of the detection of the faint bands that could have been poorly detected in the smaller
688 gel (i.e., protein bands appear sharper in bigger gel).
689
690 It is extremely important to choose proper SDS-PAGE gel to map post-translational modifications
691 (PTMs) of a specific protein thorough LC-MA/MS. The most widely used gel system for SDS-PAGE
692 is the Laemmli system, which uses Tris-glycine gels comprising a stacking gel component and the
693 resolving gel component. In this classical system, the pH and ionic strength of the buffers used in
694 the stacking gel (Tris, pH 6.8) and resolving gel (Tris, pH 8.8) are different from the buffer used
695 for running the gel (Tris, pH 8.3). Band distortion, loss of resolution, or artifact bands may be
696 caused by the highly alkaline operating pH of the Laemmli system. The previous report described
697 major causes of poor band resolution with the Laemmli system[16], including instability and short
698 expiration period of the resolving gel due to hydrolysis of polyacrylamide at the high pH, chemical
699 alterations of sample proteins, reoxidation of reduced disulfides of cysteine residues of proteins,
700 and cleavage of Asp-Pro bonds of proteins with heating at 95-100 °C in Laemmli sample buffer at
701 pH 5.2. The commercially available 4%-12% Bis-Tris protein gels are Bis-Tris HCl–buffered (pH 6.4)
702 and operated at pH ca. 7.0 unlike traditional Tris-glycine gels [16]. The numerous merits comparing

with the Laemmli system are generated by the neutral operating pH of the Bis-Tris systems [16], including high stability and long expiration period of the resolving gel, enhanced sample protein stability during electrophoresis at neutral pH leading to sharper band resolution and accurate results, and complete reduction of disulfides and absence of cleavage of Asp-Pro bonds. In this report, we could successfully map PTMs of EYFP-CENP-A K124R protein (**Figure 3**) using the commercially available 4%-12% Bis-Tris protein gels. Therefore, the commercially available 4%-12% Bis-Tris protein gels are highly recommended to use for this purpose.

For mapping post-translational modifications (PTMs) of a specific protein, in-gel digestion of target protein coupled with LC-MS/MS is widely used. In this study, we sought to identify ubiquitination sites EYFP-CENP-A K124R using affinity purification-mass spectrometry (AP-MS) strategy. Therefore, the first key step is to obtain a large amount of ubiquitinated EYFP-CENP-A K124R protein with high purity using immunoprecipitation from EYFP-CENP-A K124R overexpressing cells, which could greatly facilitate the identification and confirmation of ubiquitination sites of EYFP-CENP-A K124R by LC-MS/MS. To reduce the interference of non-ubiquitinated EYFP-CENP-A K124R protein, western blots of anti-GFP, anti-HA (Ub), anti-Ubiquitin (see section [6.1.6]), and Coomassie blue staining (see section [6.1.7]) were performed to precisely determine the position of ubiquitinated EYFP-CENP-A K124R on SDS-PAGE gel. Lastly, only a minimized area of gel band containing ubiquitinated EYFP-CENP-A K124R was excised for in-gel digestion coupled with LC-MS/MS to get optimized results. When the dried peptides are reconstituted before operating LC-MS/MS, 3% (v/v) acetonitrile /2% (v/v) formic acid was used to help better solubility and recovery rate of peptides. Sometimes the database searching could result in PTMs which does not truly exist due to the approximate assignment algorithm of search engine. Thus, another critical step is to confirm EYFP-CENP-A K124R ubiquitination sites *via* manually reviewing MS/MS of ubiquitinated peptides with help of Software F (**Table of Materials**), which could eliminate "unreal" ubiquitination sites introduced by approximate assignment by database searching. In summary, this AP-MS strategy has established an efficient and robust pipeline for the identification of EYFP-CENP-A K124R ubiquitination sites with crucial biological significance. More importantly, this pipeline could also be widely extended to investigate the PTMs of various functional proteins.

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DISCLOSURES:

The authors declare no competing interests.

REFERENCES:

1 Dunleavy, E. M. et al. HJURP is a cell-cycle-dependent maintenance and deposition factor of CENP-A at centromeres. *Cell.* **137** (3), 485-497 (2009).

2 Foltz, D. R. et al. Centromere-specific assembly of CENP-a nucleosomes is mediated by HJURP. *Cell.* **137** (3), 472-484 (2009).

3 Bernad, R. et al. Xenopus HJURP and condensin II are required for CENP-A assembly. *J Cell Biol.* **192** (4), 569-582 (2011).

4 Niikura, Y. et al. CENP-A K124 Ubiquitylation Is Required for CENP-A Deposition at the Centromere. *Developmental Cell.* **32** (5), 589-603 (2015).

5 Niikura, Y., Kitagawa, R., Kitagawa, K. CENP-A Ubiquitylation Is Inherited through Dimerization between Cell Divisions. *Cell Reports.* **15** (1), 61-76 (2016).

6 Fachinetti, D. et al. CENP-A Modifications on Ser68 and Lys124 Are Dispensable for Establishment, Maintenance, and Long-Term Function of Human Centromeres. *Developmental Cell.* **40** (1), 104-113 (2017).

7 Niikura, Y., Kitagawa, R. Kitagawa, K. CENP-A Ubiquitylation Is Required for CENP-A Deposition at the Centromere. *Developmental Cell.* **40** (1), 7-8 (2017).

8 Niikura, Y., Kitagawa, R., Fang, L., Kitagawa, K. CENP-A Ubiquitylation Is Indispensable to Cell Viability. *Developmental Cell.* **50** (6), 683-689 (2019).

9 Srivastava, S., Foltz, D. R. Posttranslational modifications of CENP-A: marks of distinction. *Chromosoma.* **127** (3), 279-290 (2018).

10 Srivastava, S., Zasadzinska, E., Foltz, D. R. Posttranslational mechanisms controlling centromere function and assembly. *Current Opinion in Cell Biology.* **52**, 126-135 (2018).

11 Ohkuni, K., Takahashi, Y., Basrai, M. A. Protein purification technique that allows detection of sumoylation and ubiquitination of budding yeast kinetochore proteins Ndc10 and Ndc80. *Journal of Visualized Experiment.* (99), e52482 (2015).

12 Niikura, Y. et al. CENP-A K124 Ubiquitylation Is Required for CENP-A Deposition at the Centromere. *Developmental Cell.* **32** (5)589- 603 (2015).

13 Niikura, Y., Kitagawa, K. Immunofluorescence Analysis of Endogenous and Exogenous Centromere-kinetochore Proteins. *Journal of Visualized Experiment.* (109), e53732 (2016).

14 Lamb, J. R., Tugendreich, S. & Hieter, P. Tetratrico peptide repeat interactions: to TPR or not to TPR? *Trends in Biochemical Sciences.* **20** (7), 257-259 (1995).

15 Leopold, A. V., Chernov, K. G., Verkhusha, V. V. Optogenetically controlled protein kinases for regulation of cellular signaling. *Chemical Society Reviews.* **47** (7), 2454-2484 (2018).

16 Inc., T. F. S. *Protein gel electrophoresis technical handbook*, <https://www.thermofisher.com/content/dam/LifeTech/global/Forms/PDF/protein-gel-electrophoresis-technical-handbook.pdf> (2015).

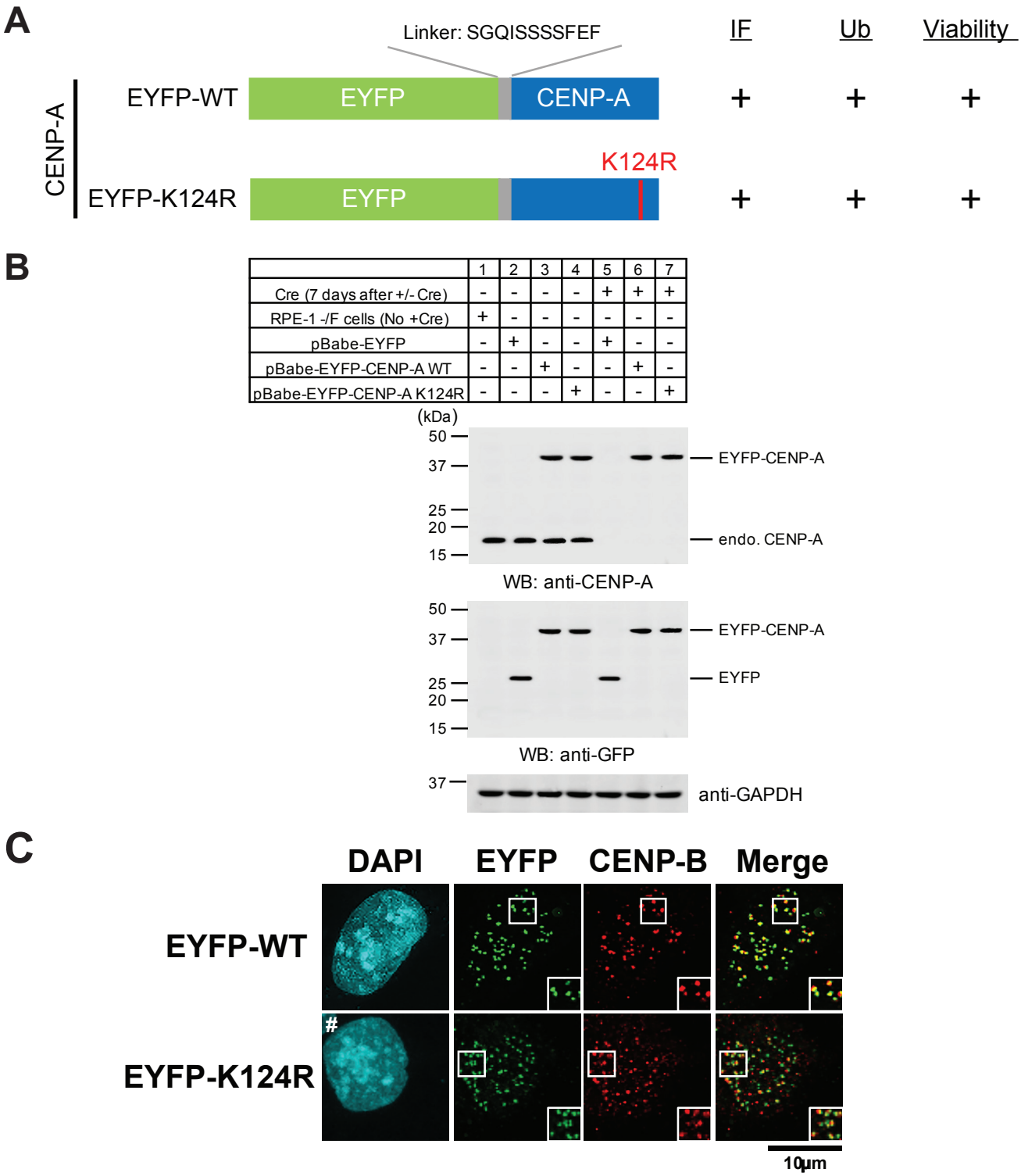
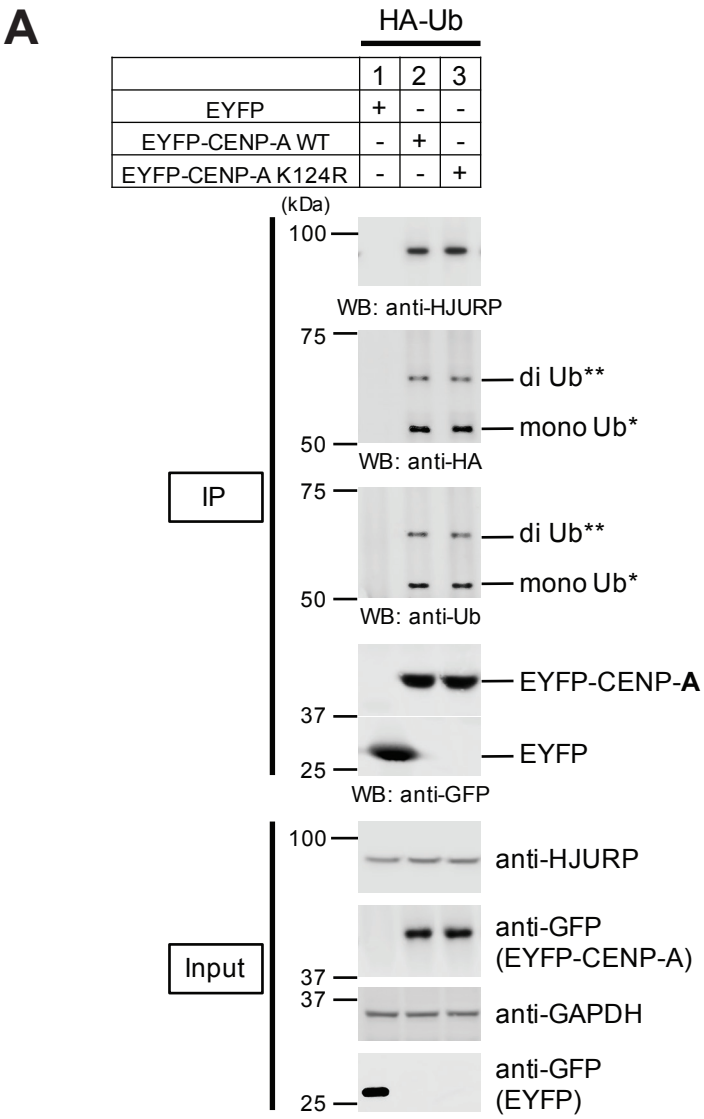
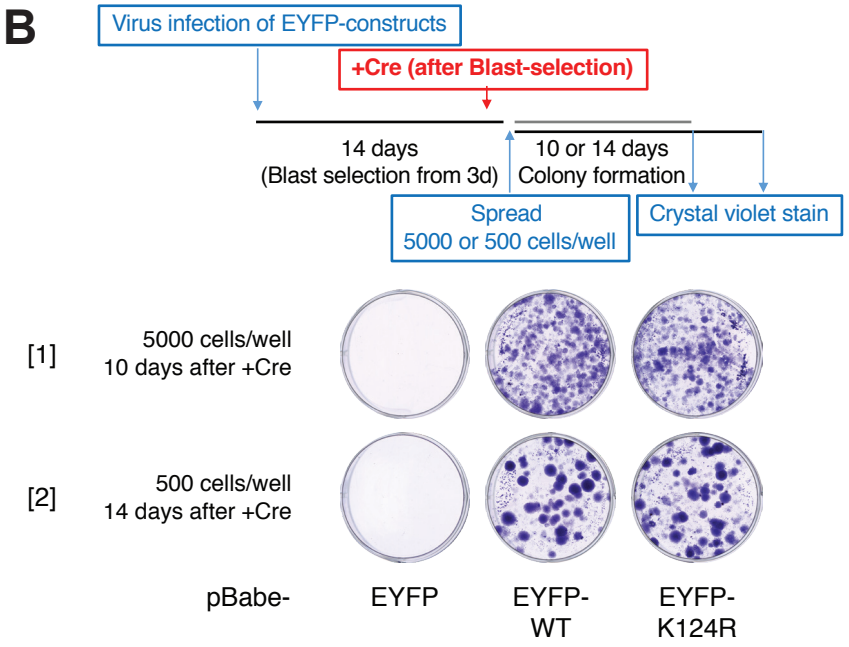


Figure 1

Figure 2





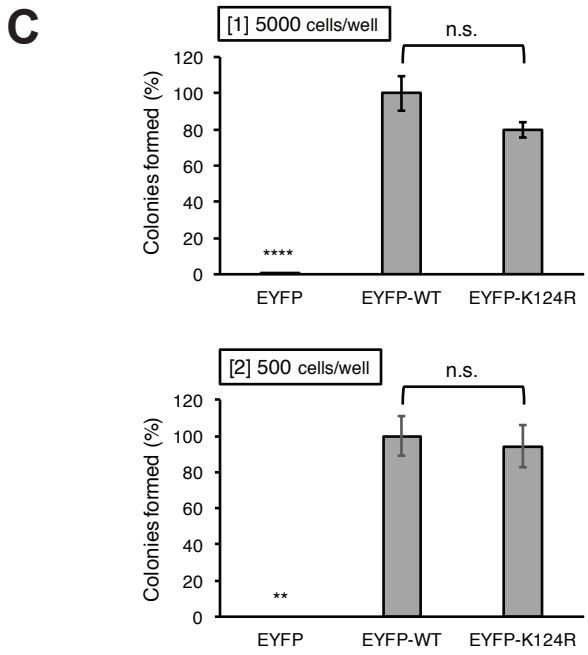
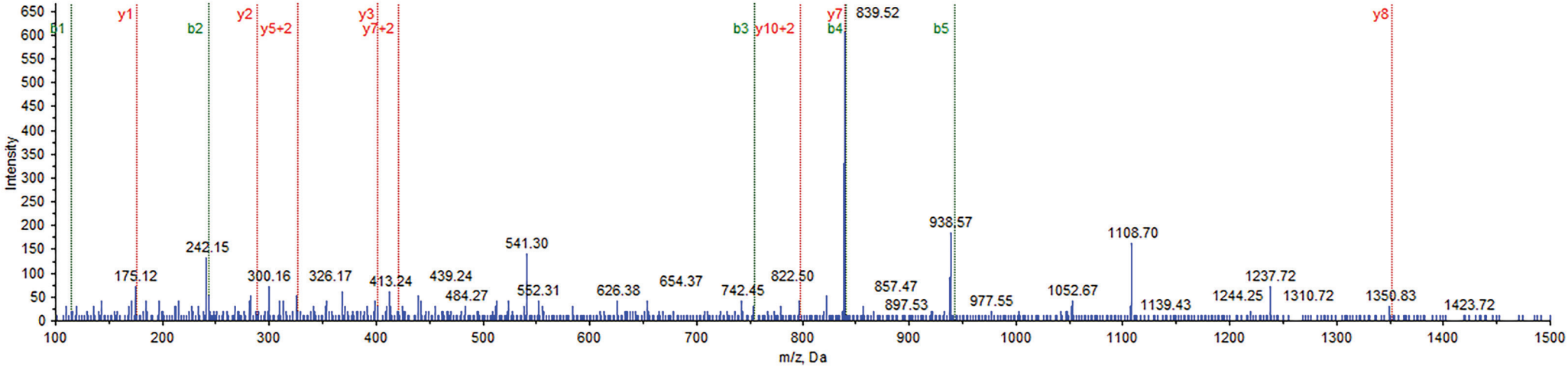


Figure 2

Figure 3

A

K306 (K56 in endogenous CENP-A), **LQK^{LRGG}STHLLIR**, Coverage 95.9%, Confidence 87.8%



B

Residue	b	b+2	y	y+2
L	114.0913	57.5493	1591.9755	796.4914
Q	242.1499	121.5786	1478.8914	739.9493
K[Umc]	753.4730	377.2401	1350.8328	675.9201
S	840.5050	420.7561	839.5098	420.2585
T	941.5527	471.2800	752.4777	376.7425
H	1078.6116	539.8094	651.4301	326.2187
L	1191.6957	596.3515	514.3711	257.6892
L	1304.7797	652.8935	401.2871	201.1472
I	1417.8638	709.4355	288.2030	144.6051
R	1573.9649	787.4861	175.1190	88.0631

Figure 2

Table 1. Plasmid vectors used in this study.

B number	Relevant characteristic(s)	Reference
B3027	pBabe-puro-Cre	Niikura et al., 2019
B3182	pBabe-EYFP	Niikura et al., 2019
B3161	pBabe-EYFP-CENP-A WT	Niikura et al., 2019
B3164	pBabe-EYFP-CENP-A K124R	Niikura et al., 2019
B3031	psPAX2	Niikura et al., 2019
D3032	pMD2.g	Niikura et al., 2019
B3189	pGP	Niikura et al., 2019
B3190	pCI-VSVG	Niikura et al., 2019
B3001	pPAM	Niikura et al., 2019
B3252	pQCXIP-EYFP	Niikura et al., 2019
B3254	pQCXIP-EYFP-CENP-A WT	Niikura et al., 2019
B3256	pQCXIP-EYFP-CENP-A K124R	Niikura et al., 2019
B2806	pCGN-HA-Ubiquitin	Niikura et al., 2019

Source
Dr. Amruta Ashtekar, Dr. Lawrence S. Kirschner
-
Dr. Daniele Fachinetti, Dr. Don W. Cleveland
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Dr. John Thompson, Dr. Gustavo W. Leone
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Dr. Kenji Tago (Jichi Medical Univeristy, Japan)
Dr. Kenji Tago (Jichi Medical Univeristy, Japan)
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Table 2. Combinations of helper/packaging plasmid vectors used in (1.1.3): Retroviru

B number	Helper/packaging plasmid vectors	Combination 1	Combination 2
B3031	psPAX2 (lentiviral gag/pol vector)	2μg	
D3032	pMD2.g (lentiviral env vector)	2μg	
B3189	pGP (retroviral gag/pol vector)		2μg
B3190	pCI-VSVG (retroviral env vector)		2μg
B3001	pPAM (amphotropic helper vector encoding retroviral gag-pol-env)		

ıs transfection of pBabe-EYFP-CENP-A constructs.

Combination 3
2μg

Table of Materials

Click here to access/download;Table of Materials;Table of Materials_YN21=final.xlsx

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Equpiments/Tools			
0.5 ml protein low binding tubes	Eppendorf	022431064	For mass spectrometry analysis
10cm cell culture dish	BIOFIL/JET, China	700224	10 cm tissue culture dish (Yohei lab, PN63)
6 Well Cell Culture Cluster	Fisher/Corning Incorporated	07-200-83	6-well culture plate
CentriVap	LABCONCO	-	Benchtop vacuum concentrator for vaccum dry peptides for mass spectrometry analysis
ChromXP C18CL, 120A, 15 cm x 75 µm	Eksigent Technologies	805-00120	Liquid chromatography (RPLC) column for mass spectrometry analysis
HCX PL APO 100x oil immersion lens	Leica	LEICA HCX PL APO NA 1.40 OIL PHE	100X Oil immersion lens
HCX PL APO 63x oil immersion lens	Leica	LEICA HCX PL APO NA 1.40 OIL PH 3 CS	63X Oil immersion lens
Immobilon-FL PVDF Transfer Membrane	EMD Millipore	IPVH00010	For western blot
Leica DM IRE2 motorized fluorescence microscope	Leica	-	motorized fluorescence microscope
Leica EL6000 compact light source	Leica		External light source for fluorescent excitation
Micro Cover glass (22 mm x 22 mm)	Surgipath	105	Cover glass (22 mm x 22 mm)
Model V16-2 polyacrylamide gel electrophoresis apparatus	Apogee Electrophoresis/CORE Life Sciences	31071010	Gel electrophoresis apparatus I to apply bigger SDS-PAGE gel
nanoLC.2D	Eksigent Technologies	-	liquid chromatography system for mass spectrometry analysis
NuPAGE 4%-12% Bis-Tris Protein Gels	Thermo Fisher	NP0335BOX	The commercially available 4%-12% Bis-Tris protein gels for mass spectrometry analysis
Olympus FLUOVIEW FV3000 confocal laser scanning microscope	Olympus	-	Confocal laser scanning microscope (https://www.olympus-lifescience.com.cn/en/support/downloads/#!dlOpen=%23detail847250519)
ORCA-R2 Degital CCD camera	Hamamatsu	C10600-10B	CCD camera
PAP Pen	Binding Site	AD100.1	For a water repellant barrier in immunofluorescent staining
TISSUE CULTURE DISHES 10CM	VWR	25382-166	10 cm tissue culture dish
Vertical electrophoresis for gel running (big size)	Junyi, China	JY-SCZ6+	Gel electrophoresis apparatus II to apply bigger SDS-PAGE gel (Yohei lab, PE23)
VWR Micro Slides, Frosted	VWR International	48312-013	Micro slides
Primary antibodies			
Anti-CENP-A antibody	Stressgen/Enzo Life Sciences	KAM-CC006	Mouse monoclonal antibody
Anti-CENP-B antibody	Novus Biologicals	H00001059-B01P	Mouse monoclonal antibody

anti-GAPDH	ABCAM	ab37168	Rabbit polyclonal antibody
anti-GAPDH	Invitrogen	PA1987	Rabbit polyclonal antibody
anti-GFP antibody	ANTI #76 (Homemade antibody)		Rabbit polyclonal antibody
anti-HA (3F10)	Roche	11815016001	Rat monoclonal antibody
anti-HJURP	Proteintech Group	15283–1-AP	Rabbit polyclonal antibody
anti-Ubiquitin	Bethyl Laboratories	A300-317A-1	Rabbit polyclonal antibody
Reagents			
Bio-Rad Protein Assay	Bio-Rad	500-0006	Commercial protein assay reagent I for measurement of protein concentration (compatible with 0.1% SDS)
Branson SONIFIER 450			Sonicator
Branson Ultrasonics sonicator Microtip Step, Solid, Threaded 9.5 mm	VWR Scientific Products Inc.	33995-325	Disruptor horn for sonication
Branson Ultrasonics sonicator Microtip Tapered 6.5 mm	VWR Scientific Products Inc.	33996-185	Microtip for sonication
Buffer A1	-	-	20 mM Tris-HCl, pH 7.4; 50 mM NaCl; 0.5% Nonidet P-40; 0.5% deoxycholate; 0.5% SDS; 1 mM EDTA; complete EDTA-free protease inhibitor reagent
Complete EDTA-free protease inhibitor cocktail	Roche	11-873-580-001	Complete EDTA-free protease inhibitor reagent for buffer A1
Coomassie brilliant blue R-250	BBI Life Sciences	CAS 6104-59-2	Coomassie blue solution for mass spectrometry analysis
Crystal violet solution (2.3% crystal violet, 0.1% ammonium oxylate, 20% ethanol)	SigmaI-Aldrich	HT90132-1L	For colony staining
DAPI	SIGMA-SLDRICH	D9542	For nuclear staining
DMEM: F12 Medium	ATCC	30-2006	DMEM: F12 Medium
Fetal Bovine Serum, certified, heat inactivated, US origin	Life Technologies/Gibco	10082	FBS (fetal bovine serum)
High-glucose DMEM (Dulbecco's modified Eagle's medium)	Life Technologies/BioWhittaker	12-604	high-glucose DMEM
Lipofectamin 3000	Life Technologies/Invitrogen	L3000	Transfection reagent I for chemical transfection
Lipofectamin 3000, P3000 solution	Life Technologies/Invitrogen	L3000	Transfection reagent II for chemical transfection
Methanol	Fisher	A412-4	Fixation reagant
Non fat powdered milk (approved substitution for carnation powdered milk)	Fisher Scientific	NC9255871 (Reorder No. 190915; Lot# 90629)	Non-fat skim milk
Opti-MEM I	Life Technologies/Invitrogen	31985	Reduced serum media
p-phenylenediamine	SIGMA-SLDRICH	P6001	For mounting medium
Penicillin, Streptomycin; Liquid	Fisher/Gibco	15-140	Penicillin-streptomycin
Poly-L-Lysine SOLUTION	SIGMA-SLDRICH	P 8920	Poly-L-Lysine, 0.1% w/v, in water

Polyethyleneimine [PEI]; 1.0 mg/ml	Polysciences	23966–2	Transfection reagent III for chemical transfection
Protein A sepharose CL-4B beads	GE Healthcare/Amersham	17-0963-03	Protein A sepharose CL-4B beads for in vivo ubiquitylation assays using pQCXIP-EYFP-CENP-A
Restore Western Blot Stripping Buffer	Thermo Scientific	PI21059	Western Blot Stripping Buffer I
Sequencing grade trypsin	Promega	V5111	For mass spectrometry analysis
SuperSignal West Femto Maximum Sensitivity Substrate	Thermo	34095	Ultra-sensitive enhanced chemiluminescent (ECL) substrate
UltraPure Distilled Water	Life Technologies/Invitrogen/Gibco	10977	Sterile tissue culture grade water
Western Blot Stripping Buffer II ((50 mM Tris-HCl, pH 6.85; 2% SDS; 50 mM DTT; 100 mM 2-Mercaptoethanol)	-	-	Western Blot Stripping Buffer II
Secondary antibodies			
Alexa Fluor 488 Goat Anti-Rabbit IgG	Life Technologies/Invitrogen	A11008	fluorophore-conjugated secondary antibody (Affinity-purified secondary antibody)
Alexa Fluor 594 Goat Anti-Mouse IgG	Life Technologies/Invitrogen	A11005	fluorophore-conjugated secondary antibody (Affinity-purified secondary antibody)
Softwares			
Acquisition FV31S-SW software	Olympus	-	Sofware C1 (https://www.olympus-lifescience.com.cn/en/support/downloads/#!dlOpen=%23detail847250519)
Analysis FV31S-DT software	Olympus	-	Sofware C2 (https://www.olympus-lifescience.com.cn/en/support/downloads/#!dlOpen=%23detail847250519)
cellSens Dimension software Ver. 1.18	Olympus	-	Sofware C3 (https://www.olympus-lifescience.com.cn/en/software/cellsens/)
Image Studio Analysis Software Ver 4.0	LI-COR Biosciences	-	Software D
Molecular Imager Versadoc MP4000 System	Bio-Rad	-	Chemiluminescence imager for immunoblot detection
Odyssey CLx Infrared imaging System	LI-COR Biosciences	-	Infrared imaging system for immunoblot detection
OpenCFU saftware	-	-	For colony counting (http://opencfu.sourceforge.net/)
Openlab version 5.5.2. Scientific Imaging Software	Improvision/PerkinElmer	-	Software A
ProteinPilot Software version 4.5	AB SCIEX	-	Software F for mass spectrometry analysis
Quantity One 1-D analysis software	Bio-Rad	-	Software E

TripleTOF 5600+ System	AB SCIEX	-	Mass spectrometry instrument
Volocity version 6.3 3D Image Analysis Software (Volocity Acquisition)	PerkinElmer	-	Software B1
Volocity version 6.3 3D Image Analysis Software (Volocity Quantification)	PerkinElmer	-	Software B2

Rebuttal letter (Answers to reviewer's comment)

Page 0

TITLE: Manuscript needs a thorough proofreading.

Yes, we have done it.

Page 3

PROTOCOL: Please remove the redundancy from the protocol. Please make the steps crisps and write as if you are describing someone how to perform your experiment with all specific action associated with it.

Yes, we removed the redundancy, made the steps and wrote as if we are describing someone how to perform our experimnet with all specific action associated with it.

Please describe all the steps in order. Presently it is very confusing. All transfections can be combined in one section then one can go from there.

Yes, we described all the steps in order. We noticed that the previous 2.3 should be shifted into Protocol 1, and now we corrected it.

Protocol needs more clarity.

Yes, we made it clearer.

The link between each section of the protocol is missing. Please bring out clarity.

Yes, we made more link between each section of the protocol.

Also please avoid going beyond 3 substep – 1 is followed by 1.1 and then 1.1.1.

Steps were written in order. All protocols and link between each section is sufficiently fulfilled.

We avoided beyond 3 steps except Supplemental Code Files which is inevitable, and such manner was accepted in the previous our manuscript [1].

1.1.3. This is same as the note so can be removed.

Now your comment shown here was shifted from Protocol 2 to Protocol 1, together with copy/paste of my text. Thus, we keep this note in the new Protocol 1 and remove the note in the new Protocol 4.

1.1.4. Which plasmid is used here?

Now we clearly described it.

Page 4

1.1.7. Is this DNA or RNA mix?

It was our mistake that we left "RNA". Now we clearly described it as "DNA-lipid complex".

1.2. From step?

1.2.1 From step?

If I did not misunderstand this comment, does this ask what the previous step 2.3.3.(the new steps 1.2 and 1.2.1) is from? I believe it is clear now that the new step 1.2 is after 1.1.9, and we also clearly explained about CENP-A$^{-/F}$ RPE-1 cells in the NOTE of Protocol 1. The

steps 1.2 and 1.2.1 are “initial steps” for Retrovirus infection of CENP-A$^{-/F}$ RPE-1 target cells starting from the spreading of these target cells.

1.2.1. Culture plate?
We corrected it to “6 well cell culture plate”. We also corrected “6-well polystyrene plate” to “6 well culture plate” in the new steps 1.1.7 and 2.2.1, and Table of Materials to be consistent.

1.2.1. Please clarify or reword.
We eliminated “sufficiently”.

1.2.2. From step?
We described “collect supernatant containing virus of (1.1.9)”.

Page 5
2.2. What is the difference between step 1 and 2.2? You already performed the transfection in step 1. Same plasmid?
Yes, now we shifted the transfection step in Protocol 2 from Protocol 1 to avoid redundancy.

2.2.1. Constructs or cell line containing pBabe-EYFP-CENP A? please clarify. Still unable to differentiate between step 1 and 2.2 and 2.3? If these are same, then maybe describe the transfection in step 1 and immunofluorescence in step 2? Needs more clarity.
We corrected the previous 2.3. Now this becomes the new 2.2.1. Previous 2.2.1 is now put as the “NOTE” below the new 2.2.1..

2.4. Which cells from which step? Please bring out clarity.
The previous 2.4 becomes the new 2.3.1.. In this way, it is now clearer that the new 2.3.1 follows the new 2.2.1. As a consequence, the previous 2.5s are now 2.4s.

Page 6
4.1. This transfection was already performed in step 1,2? Mention of three construct
Yes, now we corrected as the new (4.1.1). The 3 constructs are now mentioned in 1.1.2..

Page 8
6.1. What is the significance of doing this? Please bring out clarity with respect to what is the difference between each plasmid and why it is used somewhere in the introduction or before the start of the step.
We noted the significance and brought out the clarity under the NOTE of the Protocol 6.

6.1.3.1. Please make complete sentence and do not make points.
We corrected the sentence as follow:
NOTE: The pCGN-HA-Ubiquitin (B2806) vector is added to all samples of (i)-(iii).

Page 9
6.2.3. Based on?

The phrase: "*based on the efficiency of the immunoprecipitation*" is now added.

Page 10

6.3.3. Notes cannot be filmed so please do not highlight.

Yes, we eliminated yellow color from the NOTEs.

Page 11

7.2.1. This subheading is redundant as can be removed.

Yes, we removed it.

Page 12

7.2.10. Please divide into two steps.

Yes, we divided.

7.2. (Removed)

Made all the button clicks as bold please check.

Yes, we made so and checked it.

7.2.13. Reworded to bring out clarity please check.

Yes, we reworded and brought out clarity.

7.2.14. What is the proper folder?

We removed this remark.

7.2.15) How is this done?

We confirmed the searching was done against regular database, so we deleted the description "concatenated with reverse decoy database".

Page 13

Citation?

Yes, we added the citation.

Page 16

Xxx cells expressing EYFP-CENP-A

It was missing "in" before "the RPE-1 CENP-$A^{-/F}$ cells". We corrected as this:

"*using EYFP-CENP-A K124R expressed in the RPE-1 CENP-$A^{-/F}$ cells* ".

References

1 Niikura, Y. & Kitagawa, K. Immunofluorescence Analysis of Endogenous and Exogenous Centromere-kinetochore Proteins. *J Vis Exp.* (109), e53732, doi:10.3791/53732, (2016).

SUPPLEMENTAL CODING FILES

2.6.1) Open Software A (**Table of Materials**) on the desktop. For using Software A;

2.6.1.1) For basic commands of document management;

2.6.1.1.1) Select File > New > Image Document to open LIFF file (file of multilayered documents). Note that if you can not see the LIFF file on the screen, select Window > Palettes and see “untitled layers” or the file name of the LIFF file you designated, which usually appears at the bottom in the Palettes options. This command is useful to find any working palettes on the screen.

2.6.1.1.2) To save LIFF file (file of multilayered documents), select File > Save As. Choose Format: Openlab 5 LIFF. To open LIFF file, select File > Open.

2.6.1.1.3) To save single or multiple layer(s) in LIFF file as TIFF file format, select File > Save As Multiple. Choose Format: TIFF for publication. To open single or multiple TIFF file(s), select File > Open Multiple.

2.6.1.1.4) To crop layer, select Image > R.O.I. > Crop Document. Save LIFF file or TIFF file of layer as described in (2.6.1.1.2) and (2.6.1.1.3).

2.6.1.1.5) To make merged image, select specific layers to be merged in the layer palette. Drug these layers together on the "New" button at the top of the layer palette. The merged image appears at the bottom of the layer palette (i.e., LIFF file).

2.6.1.1.6) To create new State Server Palette or edit pre-existing State Server Palette, select Windows > Palettes > sate server. To create new State Server Palette, click on the New button on the State Server Palette. Check the items in the scrollable list that you wish to include. Type a name for the state into the “Save as a state called:” box. Click on the Save. To edit pre-existing State Server Palette, click on the Update.

2.6.1.1.7) To show/add scale bar, first perform spatial calibration as described in (2.6.1.1.8). Select Image > Show Scale Bar. Save LIFF file or TIFF file of layer attached scale bar as described in (2.6.1.1.2) and (2.6.1.1.3).

2.6.1.1.8) To perform spatial calibration;

2.6.1.1.8.1) Load a reference image that contains an object of a known size, for example a calibration slide with a graticule of known dimension.

2.6.1.1.8.2) Click on the Ruler icon (which has ruler cartoon mark) in the left of the Image Document window. Click on the tab next to the right-hand arrow and adjust the

callipers so that they enclose the distance that you wish to measure: the left-hand arrow is at the start point and the right-hand arrow is at the end point.

2.6.1.1.8.3) Select Image > Calibration to display the Spatial Calibration dialog (palettes). Check that the correct unit of measurement is displayed. If necessary, select the unit that is appropriate to your measurement from the Units pop-up menu.

2.6.1.1.8.4) Click on the expander icon to expand the dialog. Click on the New to create a new entry in the settings list. It will be called untitled (1/2/3 etc.). Click in the box above the New button and type in a name for the settings. To update settings, select the appropriate entry in the scrollable list, make the required changes in the main dialog and click on the Update. To delete settings, select the entry in the scrollable list and then click on the Backspace key. The entry is deleted immediately from the scrollable list.

2.6.1.1.8.5) Check the Square Pixel Lock box so that the Y Dimension automatically defaults to the same value of the X Dimension.

2.6.1.1.8.6) Click on the Calibrate to calibrate the image. This calibration will apply to all layers in the document, and will be retained when you subsequently open the document, as long as you save it as a LIFF file. Once you have calibrated an image, all measurements on rulers and Calibration Bars will be displayed in calibrated units.

2.6.1.1.9) To import the TIFFF file image, drag directly the TIFFF file into the icon of Software A. New layer palette (i.e., LIFF file) which contains the dragged image file is created and appears.

2.6.2.1.10) To set/change color of the image file, click (select) Color Tables icon and select the required color table from the pop-up menu.

2.6.1.2) Image acquisition to capture z-stack image sequences using Software A.

2.6.1.2.1) Click on the Video Icon (which has video cartoon mark) that makes the video preview layer current so that live video can be displayed in the Document Window. You can achieve the same effect by making the video layer current in the Layer Manager Palette.

2.6.1.2.2) On the Leica CTRMIC Controller, select 0.5 µm increment (You should optimize this increment for your purpose.). Then, set bottom and set top moving z-axis Focus Drive within the interval of the layers you choose to capture.

2.6.1.2.3) Select Special > Capture Z stack to capture multiple layers with single channel. Note that Capture Z stack is a created program in an Automator Window which is available upon your request. Check on the “Capture layers every” and enter 1 µm (You

should optimize this increment for your purpose.). Enter name of captured layers, and click on the Start.

2.6.1.2.4) Select Special > Capture Multi Channel Z stack to capture multiple layers with multiple channels. Note that Capture Multi Channel Z stack is a created program in an Automator Window which is available upon your request. Enter names of Channels 1-3 (Number of channel depends on the created program in the Automator Window.). Enter state name of the State Server Palette of each channel you choose to capture layers (see [2.6.1.1.6]), and click on the Start. The captured z-stack images appear in the working layer palette (i.e., LIFF file).

2.6.1.3) Deconvolution using Software A. Note: Optionally proceed to (2.6.2.4) to perform deconvolution in Software B2 using documents acquired from Software A. Make sure that the calibration is completed for all documents in LIFF file in advance of proceeding deconvolution in Software B2.

2.6.1.3.1) Select all layers in the LIFF file you choose to apply deconvolution by clicking.

2.6.1.3.2) Select Image > Volume Deconvolution. Enter appropriate factors in Volume Deconvolution palette (e.g., Sample Z Spacing, Objective Magnification, Camera Pixel Size, Camera Binning, Medium Refr. Index, Lens Aperture, Emission Wavelength). Check Deconvolve: Selected Layers (as your option). Check “Put Results in: New Document (as your option)”. Click on the Deconvolute.

2.6.1.3.3) Choose layers of deconvulted images and drug them together into New on the top of LIFF file palettes, then the deconvoluted image of merged layers appears at the bottom in the LIFF file. Crop, save images, and attach scale bar as described in (2.6.1.1.7).

2.6.1.4) Signal quantification using Software A. Note: Optionally proceed to (2.6.2.6) to perform signal quantification in Software B2 using documents acquired from Software A.

2.6.1.4.1) Select Image > Show Measurement to open the Measurements Window. Hide one or more of the measurement options, by unchecking the boxes. You will only be able to hide a maximum of two tables. (One must always remain visible.)

2.6.1.4.2) Select Image > Start Recording to record quantified signals on the window.

2.6.1.4.3) Go back to the Image Document LIFF file, click on the ROI tool icon (top, left), and select Freehand tool. Position the mouse on the signals to be quantified, click and drag until you have selected the desired area covering the centromere-kinetochore region in image by tracking reference signals of other layer (the different channel image;

e.g., CENP-B or ACA). Release the mouse. Copy and paste Mean (Cal) value in the Measurements Window to Excel file.

2.6.1.4.4) Repeat the step of (2.6.1.4.3) to complete s_{sample}, R_{sample}, s_{ctrl}, R_{ctrl}, and b (the background signal brightness) of each, and complete calculation in Excel file as indicated in (3.3). One example of calculation was shown previously[1].

2.6.2) Open Software B1 and/or B2 (**Table of Materials**) on the desktop. For using Softwares B1 and/or B2;

2.6.2.1) For basic commands of document management for image acquisition using Software B1;

2.6.2.1.1) Select File > New Library to open new Library window.
Select File > Open Library to open the Library window. Select File > Close to close the Library window. New Library is automatically saved, when it's closed.

2.6.2.1.2) File > Export, a dialog window: "Export File" appears, determine the file name (Save As:) and folder to save (Where:). Choose Format: Item as TIFF. Click "Export". To show scale bar or select other options, click "Options...".

2.6.2.1.3) To import the image file into the Library window, select File > Import.

2.6.2.1.4) Edit > Duplicate to duplicate any files at the left tab in the Library window.

2.6.2.1.5) To create new folder, select Actions > Create New > Folder. To delete the folder, click/select the folder at the left tab in the Library window and push delete key on the keyboard.

2.6.2.1.6) To create Image Sequence file, select Actions > Create New > Image Sequence. To remove any item including Image Sequence file at the left tab in the Library window, select Actions > Remove Items.

2.6.2.1.7) To delete a specific image file inside the Image Sequence file, select "Sequence" on the top menu inside the Library window, click/select the specific image file name and push delete key on the keyboard.

2.6.2.1.8) Most of the basic settings (cannel, exposure time, binning, Gain, Offset, light intensity, Fluo Shutter, Focus Drive, Objective Turret) can be determined by the icons at the right side in the Library window.

2.6.2.1.9) Click (select) "Video Preview" at the left tab in the Library window to send video image into the main screen.

2.6.2.1.10) Click (select) “the capture single frame icon (which has camera cartoon mark)“ at the right side in the Library window to capture the single frame image in the main screen. The captured image automatically pops up at the left tab in the Library window.

2.6.2.1.11) To crop any image document file including Image Sequence file, first choose any Selection Tool (e.g., Rectangle, Freehand, Circle, Lasso, etc.) on the top menu inside the Library window as your preference. Second, position the mouse on the area to be cropped, click and drag until you have selected the desired area. Release the mouse. Third, select Actions > Crop to Selection.

2.6.2.1.12) To make merged image of different channels (of different colors; see also [2.6.2.1.16]);

2.6.2.1.12.1) Select Actions > Create New > Image Sequence to create Image Sequence file. Drag and drop an LIFF file acquired in Software A or an file acquired in Software B1 which has a set of images of different channels and/or z slices. A dialog window: “Add Planes to Sequence” appears. Input the number of “channels”, “timepoints” and “Z slices per volume”. Select “Channels then Z-slices then Timepoints”, and click “Add”.

2.6.2.1.12.2) Select “Sequence” on the top menu inside the Image Sequence file. Select multiple files of different channels inside the Image Sequence file. Select Actions > Merge Planes. The merged image file appears at the left tab in the Library window.

2.6.2.1.13) To set the Light Path Manager, click on “an unused light path manager button (Untitled light path 1, 2, 3…)" at the left tab (The Light Path Manager). The Light Path Properties dialog opens. Select your options including "Label:", "Color:", "Devices", and "Shutters". The Light Path Manager allows you to record the positions of all your automated hardware in the combinations necessary to image each fluorochrome, or channel, as well as transmitted light paths. The Light Path Manager is also useful to capture z-stack image sequences (see [2.6.2.2.1.3]).

2.6.2.1.14) To show/add scale bar, first perform spatial calibration as described in (2.6.2.1.15). Select captured image (Acquisition Protocol) at the left tab in the Library window. Select Image > Display > Show Scale.

2.6.2.1.15) To perform spatial calibration;

2.6.2.1.15.1) Load a reference image that contains an object of a known size, for example a calibration slide with a graticule of known dimension.

2.6.2.1.15.2) Click (select) the Calibration tool (ruler cartoon mark) from the toolbar. Draw a line of known length on your Video Preview. Click and hold your mouse to start the line, drag the mouse to the endpoint of your line and release the mouse button.

2.6.2.1.15.3) Select Video > Calibrate or double click on the Calibration tool to open the calibrate window. Enter line length in µm and click “Calibrate”.

2.6.2.1.16) To set/change color of the image file, select Tool > Change Colors.

2.6.2.2) Image acquisition to capture z-stack image sequences using Software B1.

2.6.2.2.1) Click (select) on the “Video Preview icon (which has video cartoon mark)” that makes that live video can be displayed in the Library window before processing any commands.

2.6.2.2.1.2) Click Focus Drive icon at the right tab. Set Bottom and Set Top to determine the range of stage position to capture along z-axis.

2.6.2.2.1.3) Double click on the acquisition protocol feedback panel at the right side in the Library window, which opens the acquisition setup dialog. A dialog window: “Acquisition Setup” appears. In that window, click (select) "Cennels/Z" on the top menu. Determine the file name (Title:) Check the box: Change channels using light paths, and input “light path name (see [2.6.2.1.13])” at Channel (1, 2, 3...etc.). Add (+) and/or delete (-) channel required and/or non-required to capture images, and input “light path name” as above. Determine the option to capture with z spacing (Capture with this Z spacing:) or with number of slices (Capture this many slices:). Choose other options as your preference.

2.6.2.2.1.4) Click "OK". Click "red button (start capturing)" at the left tab. The Z slices file appears on the left tab. Export these files to the proper format (e.g., TIFF file).

2.6.2.3) For basic commands of document management for deconvolution and signal quantification using Software B2;

2.6.2.3.1) To import the image file into the Library window, select File > Import.

2.6.2.3.2) To create new folder, select Actions > Create New > Folder. To delete the folder, click/select the folder at the left tab in the Library window and push delete key on the keyboard.

2.6.2.3.3) To create Image Sequence file, select Actions > Create New > Image Sequence. To remove any item including Image Sequence file at the left tab in the Library window, select Actions > Remove Items.

2.6.2.3.4) To delete a specific image file inside the Image Sequence file, select “Sequence” on the top menu inside the Library window, click/select the specific image file name and push delete key on the keyboard.

2.6.2.3.5) To crop any image document file including Image Sequence file, first choose any Selection Tool (e.g., Rectangle, Freehand, Circle, Lasso, etc.) on the top menu inside the Library window as your preference. Second, position the mouse on the area to be cropped, click and drag until you have selected the desired area. Release the mouse. Third, select Actions > Crop to Selection.

2.6.2.3.6) To remove unnecessary z-stack slice, click (select) the image file of z-stack slices. Select Tool > Split. Choose only "necessary" z slice(s) and drag them into new Image Sequence file. Make new PSF file (see [2.6.2.4.1]) using the new Image Sequence file.

2.6.2.3.7) For any management of Image Sequence file, if you double click an specific Image Sequence file name at the left tab in the Library window, the specific Image Sequence file pops up as another window, and you could see the same menu above as the Library window.

2.6.2.3.8) To set/change color of the image file, select Tool > Change Colors.

2.6.2.4) Deconvolution using Software B2. Note that, if image documents are acquired in Software A, make sure that the calibration is completed for all documents in LIFF file in advance of proceeding deconvolution in Software B2.

2.6.2.4.1) To make a PSF file using Sofware B2;

2.6.2.4.1.1) Select File > New Library. New Library is automatically saved, when it's closed. Select Actions > Create New > Image Sequence to create Image Sequence file. Drag and drop an LIFF file acquired in Software A or an file acquired in Software B1 which has a set of images of different channels and/or z slices. A dialog window: "Add Planes to Sequence" appears. Input the number of "channels", "timepoints" and "Z slices per volume". Select "Channels then Z-slices then Timepoints", and click "Add".

2.6.2.4.1.2) Make sure to click (select) the document file to deconvolute at the left tab in the Library window. Select Actions > Create New > Calculated PSF. A dialog window: "Calculate PSF" appears.

2.6.2.4.1.3) Choose "Calculate a new PSF of this type: Widefield". Input names/values of PSF Name, Medium Ref. Index, Numerical Aperture (e.g., 60x: 1.4; 100x: 1.4; 40x: 0.6), and Emission Wavelength. Click "Create". It takes for a while to complete to calculate Widefield PSF. When the PSF calculation is completed, the PSF file icon appears at the left tab in the Library window.

2.6.2.4.2) To perform deconvolution using Sofware B2;

2.6.2.4.2.1) Skip this process if you apply pre-calculated PSF file for new deconvolution, and go to (2.6.2.4.2.2). Select Actions > Create New > Image Sequence to create Image Sequence file. Drag and drop an LIFF file acquired in Software A or an file acquired in Software B1 which has a set of images of different channels and/or z slices. A dialog window: “Add Planes to Sequence” appears. Input the number of “channels”, “timepoints” and “Z slices per volume”. Select “Channels then Z-slices then Timepoints”, and click “Add”.

2.6.2.4.2.2) Skip this process if the document file is acquired in Software B1, and go to (2.6.2.4.2.3). If the document file is acquired in Software A, select Edit > Properties. Input values of “µm/pixel (X)”, “µm/pixel (Y)”, and “µm/pixel (Z)”. For XY, input the values following information available in the Spatial Calibration dialog (see [2.6.1.1.8]). For Z, input the interval of the Z-section. Input: Microscope Objective, and click “Change”.

2.6.2.4.2.3) Make sure to click (select) the document file to deconvolute at the left tab in the Library window.

2.6.2.4.2.4) Select Tools > Fast Restoration or Iterative Restoration. In case of Iterative Restoration, input Confidence limit: 100%, Iteration limit 20%. Select PSF from the pop-up menu. Click “Start”. It takes for a while to complete the deconvolution process. When the deconvolution is completed, click “Done” in the restoration window.

2.6.2.4.2.5) Check the deconvoluted image at the right tab in the Image Sequence file (by clicking green icon). To optimize performance of the deconvolution, try cropping the Image Sequence file choosing optimized area (see [2.6.2.3.5]) or try removing unnecessary z slice (see [2.6.2.3.6]), make a PSF file, and perform deconvolution.

2.6.2.5) Edition process after the deconvolution using Sofware B2;

2.6.2.5.1) Skip this process if you don’t export the deconvoluted images into the LIFF file of Software A, and go to (2.6.2.5.2). Make sure to click (select) the “deconvoluted” Image Sequence file at the left tab in the Library window. Select “Sequence” on the top menu inside the Library window, click/select the specific name of the “deconvoluted” Image Sequence file. Or perform the same process using commands as describe in (2.6.2.3.6).

2.6.2.5.1.2) Select Actions > Merge Planes. The “Merged” Image Sequence file appears at the left tab in the Library window.

2.6.2.5.1.3) Select this “merged” Image Sequence file at the left tab in the Library window. Select Tools > Split Volumes to split the volumes of this “merged” Image Sequence file into the separate z slices of images. Choose your option in the dialog

window: Split Volumes, then click “Split”. The “Merged-split” Image Sequence folder appears at the left tab in the Library window.

2.6.2.5.1.4) Inside this folder, select the “merged-split” Image Sequence file. Select File > Export. Determine the file name and save location, and click “Export”. Make sure the “.liff” extension of the exported file. Open the exported file by Sotware A. Select images in the Layer Manager Palette. Perform image file edition using Software A.

2.6.2.5.2) Optionally, select Edit > Duplicate the Image Sequence file containing the deconvoluted images, and/or select File > Export to any type of format (e.g., LIFF file, TIFF file, etc.) and edit images on that format.

2.6.2.6) Signal quantification using Software B2.

2.6.2.6.1) Select File > New Library. New Library is automatically saved, when it’s closed.

2.6.2.6.2) Select Actions > Create New > Image Sequence to create Image Sequence file. Drag and drop an LIFF file acquired in Software A or an file acquired in Software B1 or B2 which has a set of images of different channels.

2.6.2.6.3) A dialog window: “Add Planes to Sequence” appears. Input the number of “channels”, “timepoints” and “Z slices per volume”. Select “Channels then Z-slices then Timepoints”, and click “Add”.

2.6.2.6.4) Click “Measurements” on the top menu inside the Library window. You can change Select Freehand and position the mouse on the signals to be quantified, click and drag until you have selected the desired area covering the centromere-kinetochore region in image by tracking reference signals of the different channel image (e.g., CENP-B or ACA). Release the mouse.

2.6.2.6.5) Change “Tile Channels” status (top, left in the Library window) to show multiple channel images and make sure the selected area is corresponding among all images of the different channels.

2.6.2.6.6) The table of measurements automatically appears at the bottom in the Library window. Copy and paste Mean value of each channel in the Measurements table to Excel file. Perform the calculation as indicated preciously [1].

4.2.1) For using the infrared imaging system;

4.2.1.1) Open Software D (**Table of Materials**) on the desktop.

4.2.1.2) To acquire images, click the Acquire tab on the top menu. Select “Auto: Intensity Auto” setting (“A” button) in Channels menu above. Click the “Start” button

(top, right) in Scanner menu above. After acquisition, image data are automatically stored in the folder selected as the Work Area when Image Studio is opened. The data are easily accessed in the Tables below the image window. This table is called Images Table.

4.2.1.3) To export and save image files, click the iS icon (the most top, the most left), then select Export > Single Image View > Current Image. “Browse” your designated folder to save image files, then “Edit Name”, and “Save”.

4.2.1.4) To quantify signal bands, click the Analysis tab on the top menu. Click the “Manual (top, left)” below the iS icon. Click the “Manual” at the bottom of further options to erase unnecessary line images on the acquired image.

4.2.1.4.1) Click the “Draw Rectangle” on the menu above. Position the mouse on the specific band to be quantified, click and drag until you have selected the desired area covering the whole region of the band. Release the mouse. Quantified values are displayed at the bottom (the location of the Images Table). Click the Shapes tab at the top left of the Table to view the data including the quantified values for each shape (rectangle). This table is called Shapes Table.

4.2.1.4.2) To save data including the quantified values for each shape (rectangle), select Report > Save As at the top-right of the Shapes Table.

4.2.2) For using the chemiluminescence imager;

4.2.2.1) Check user manual of chemiluminescence imager [2], Section 4.2.9 (not the Protocol number of the present article): Installing the Lens, for proper lens option and installation.

4.2.2.2) Open Software E (**Table of Materials**) on the desktop. Select File > VersaDoc. Click on the “Select”, then select Blotting > Chemi Ultra Sensitivity.

4.2.2.3) Click on the “Position”. During scattering of imager, position and focus the membrane incubated in working solution of an ultra-sensitive enhanced chemiluminescent (ECL) substrate (**Table of Materials**). Click on the “Stop” to stop scattering of imager after optimization of the position and the focus.

4.2.2.4) Click on the “Optimize Exposure”. Fill working values for exposure in the dialog window; Total Exposure (sec.): 300.0, Starting Exposure Time (sec.): 30.0, Number of Exposures: 10). Check “Save Images” so that sequentially captured images are automatically saved in your designated folder. Note: For optimal results, empirically determine these working values.

4.2.2.5) To capture size-marker image on the membrane, leave imager's door opened ca. 5 cm during exposure and image capturing. Repeat same process as (4.2.2.4) without changing the position and the focus of the membrane. Fill working values for exposure in the dialog window; Total Exposure (sec.): 0.6, Starting Exposure Time (sec.): 0.1, Number of Exposures: 6). Note: For optimal results, empirically determine these working values.

4.2.2.6) For size-marker image (blank image without chemiluminescence signals for size-marker positions), optionally go to Volume Quick Guide palette (or select Help > Volume Quick Guide to open that palette) and click on the "3. Transform" to open "Transform" dialog window. Check the box of "Invert display" in the window. Adjust brightness and contrast with High/Low buttons or choose Auto-scale. Click on the "OK" once optimized brightness and contrast of the image.

4.2.2.7) To export images, select File > Export to TIFF Image. To merge plural images, select View > Multi-channel Viewer.

SUPPLEMENTAL CODING FILES REFERENCES

1 Niikura, Y. & Kitagawa, K. Immunofluorescence Analysis of Endogenous and Exogenous Centromere-kinetochore Proteins. *J Vis Exp.* (109), e53732, doi:10.3791/53732, (2016).

2 Inc., B.-R. L. *VersaDoc Imaging System User's Manual*, <http://stmichaelshospitalresearch.ca/wp-content/uploads/2015/09/Bio-rad-Molecular-Imager-VersadocMP-Versadoc-Imaging-systems.pdf> (2001).

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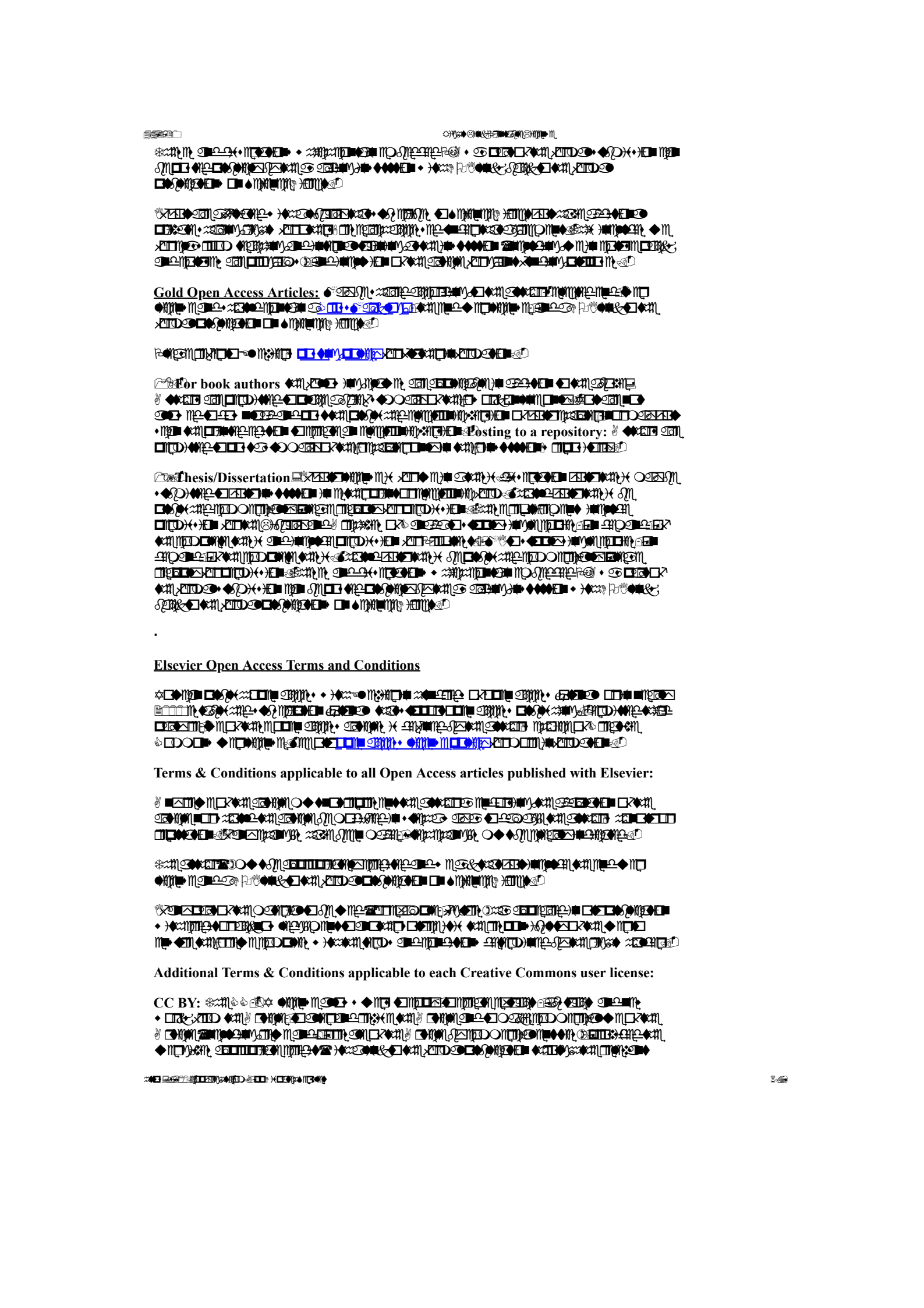

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