Video Article

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

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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 in the use of GFP/EYFP or the tagging of high molecular weight protein as a tool to analyze the function of a protein. Current technical limit is also discussed 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[1,2,3]. We have previously reported that the CUL4A-RBX1-COPS8 E3 ligase is required for CENP-A ubiquitylation on lysine 124 (K124) and centromere localization[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 suggesting that 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 problem there could 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 of CENP-A. The protein of CENP-A 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[9,10,11]. 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. Preparation of the supernatant containing retrovirus using 293T packaging cells.
    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.
    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**).
    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.
    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.
    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**).
    6. Mix solutions A and B together, and incubate at room temperature for 15 min.
    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.
    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.
    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.

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

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, **Figure 1C-E** for the data with Cre infection).
2. Perform methanol cell fixation and immunofluorescence staining as described previously[13].
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).
4. Remove excess mounting medium with a paper towel and seal the edges of the coverslip with nail polish at the last step.
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.
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).

1. Retrovirus transfection of pBabe-EYFP-CENP-A constructs.
   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.
   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. 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).
   4. 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 the results presented here).

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.
   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.
   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. 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.
   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.
   5. Perform western blotting as described in section 4 on the same day 10. Results are shown in **Figure 1B** (lanes 5-7).
   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 **Figure 1C-1E**.
   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**).
   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 **Figure 1B** and **Figure 2A** and **Table of Materials** for EYFP-CENP-A proteins.

1. Isolate proteins by lysing the cells grown with different virus infection. Then run 20 µg of protein in one well of SDS PAGE gel. Transfer the proteins to a PVDF membrane as described previously[13].

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 **Figure 1B** and **Figure 2A**.
    1. For the infrared imaging system to detect and analyze the protein bands, see step 4.2.1 in **Supplemental coding files**.
    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.
    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.

1. Transfection for in vivo ubiquitylation assays using pQCXIP-EYFP-CENP-A.
    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.
    2. Incubate the cells at 37 °C in an atmosphere of 5% CO_2 for 18 h.
    3. At 17 h after seeding, prepare the transfection reagents.
    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. 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**).
    6. Mix solutions A and B together, and incubate at room temperature for 15 min.
    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.
    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.
    9. Culture the cells at 37 °C with 5% CO_2 for 48 h after transfection. Collect cells for cell lysates preparation.

2. Preparation of protein A beads bound with anti-GFP antibody.
    1. Take 25 µL (50% v/v) of protein A beads for one reaction of immunoprecipitation (IP). Wash with buffer A1 (**Table of Materials**) at least 3x to remove EtOH, and make 50% solution 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).
    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.
    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.
    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.

3. Immunoprecipitation (IP) using protein A sepharose beads bound with anti-GFP antibody.
    1. Lyse cells obtained in step 5.1.9 in buffer A1 by sonication and freeze-thaw process.
    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.
    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.
    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. 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.
    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**.
    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

1. Transfection for mass spectrometry analysis using pQCXIP-EYFP-CENP-A.
    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 20 dishes for one immunoprecipitation (IP) sample, to obtain at least 10 mg total protein (see 5.3).
    NOTE:For optimal results, empirically determine the cell density to use in seeding.
    2. Incubate the cells at 37 °C in an atmosphere of 5% CO_2 for 18 h. Prepare the transfection reagents ~ 23 h after spreading (24 h point is 0 h point of the transfection).
    3. At 17 h after seeding, prepare the transfection reagents and transfect cells as (4.1.3). Transfected cells have pCGN-HA-Ubiquitin (B2806) and pQCXIP-EYFP-CENP-A K124R (B3256).
    4. Incubate the cells at 37 °C in an atmosphere of 5% CO_2 for 48 h after transfection. Collect cells for cell lysates.
    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. 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).
    7. Keep another sample for 90% of total immunoprecipitants for mass spectrometry analysis. Run this sample within two wells of 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.

2. Mass spectrometry analysis using in-gel digestion.
    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.
    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.
    3. Concentrate the sample using benchtop vacuum concentrator for 30 min to dry the gel pieces.
    4. Add 10 µL of 10 ng/µL sequencing grade trypsin and let the gel pieces to rehydrate for 5 min.
    5. Add 25 mM NH_4HCO_3 just enough to cover the gel pieces, digest at 37 °C overnight.
    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.
    7. Add 10 µL acetonitrile to the gel pieces, vortex 5 min, and spin down. Transfer the supernatant to the same tube.
    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.
    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**).
    10. Inject 8 µL of reconstituted sample onto a reverse phase liquid chromatography (RPLC) column.
    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.
    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.
    13. For database searching, open the commercial software (see **Table of Materials**) to analyze mass spectrometry data on the desktop.
    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.
    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/UP611385640).
    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).
    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.
    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.
    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.
    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 (**Figure 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 (**Figure 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 (**Figure 3A** and **Figure 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.

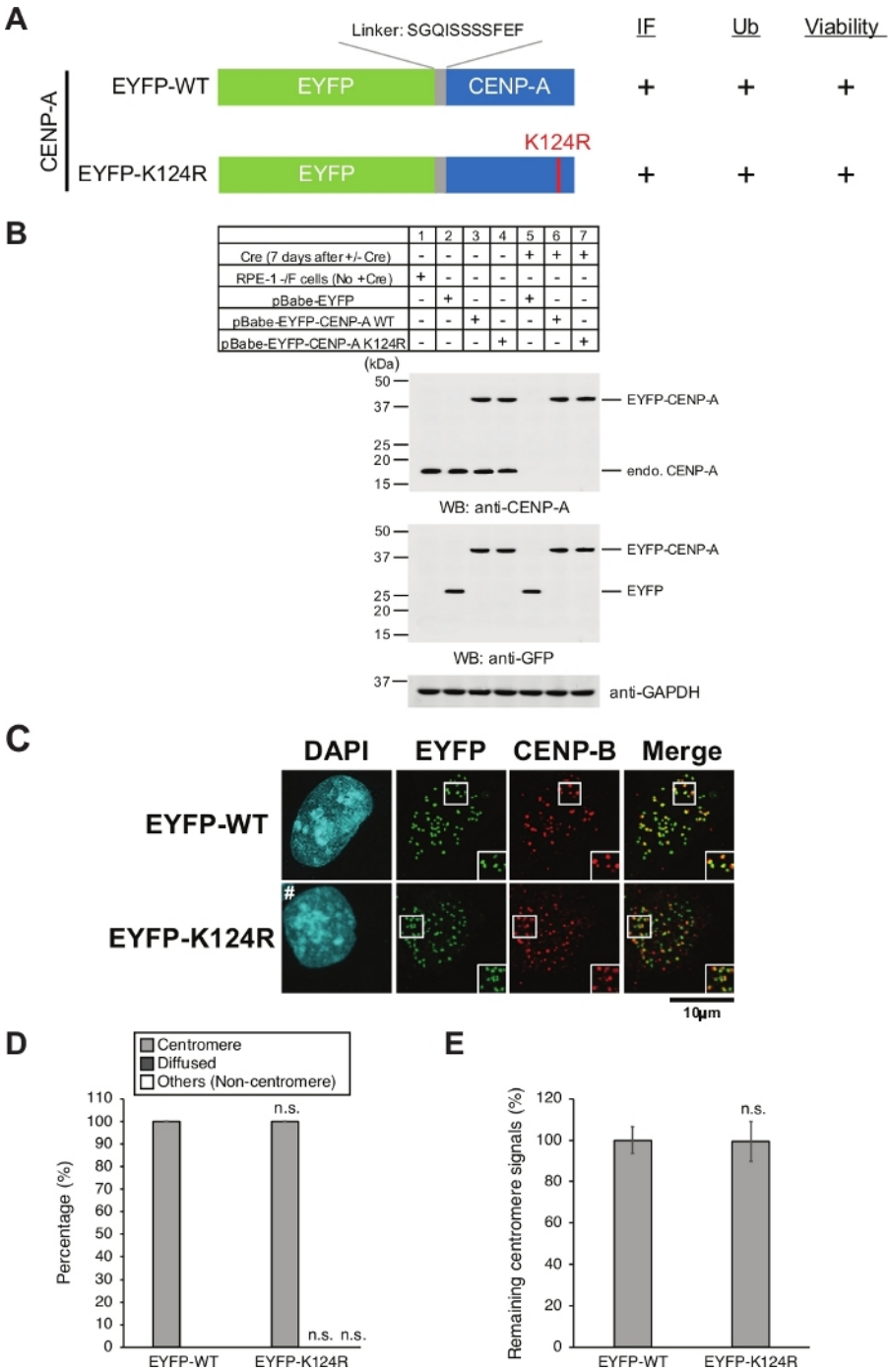


Figure 1: EYFP-CENP-A K124 mutant localizes at centromeres. (**A**) Representations of the different CENP-A protein constructs tagged with EYFP (enhanced yellow fluorescent protein) at the N terminus. Red letters mark the position of K124R amino acid substitution in the indicated construct (left). The 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 ≥ 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. Please click here to view a larger version of this figure.

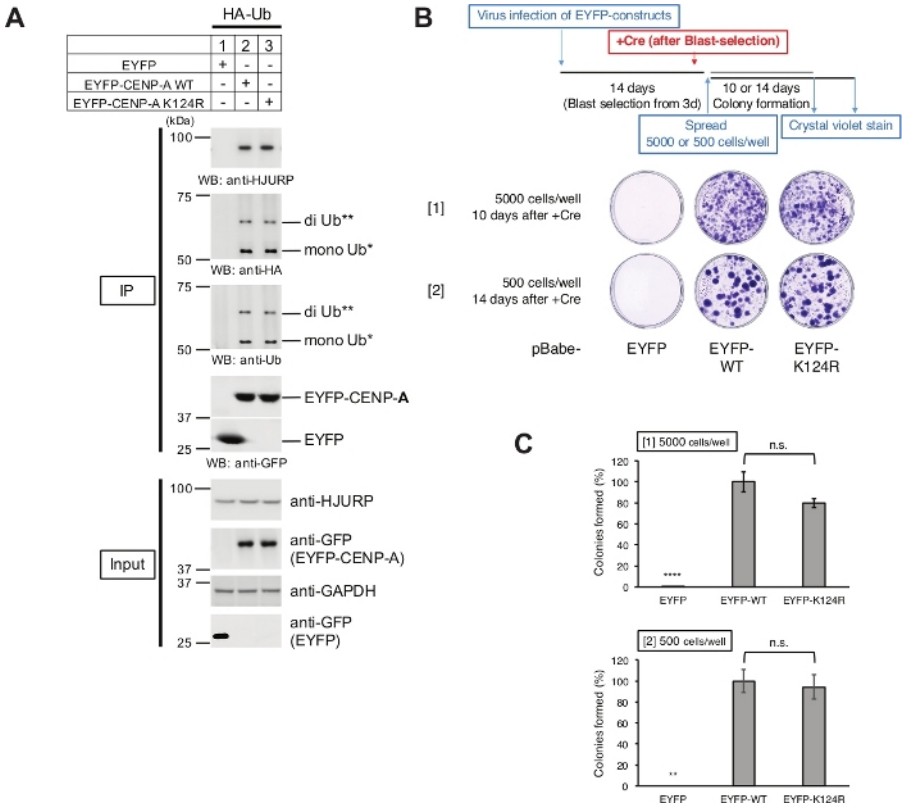
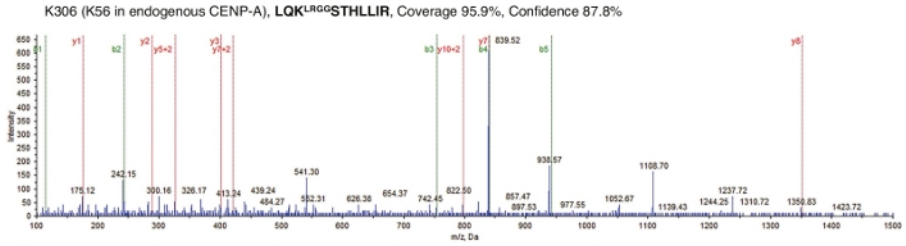


Figure 2: EYFP-CENP-A K124 mutant is ubiquitylated and interacts with HJURP, and cell viability was not affected by K124R mutation of EYFP-CENP-A. (**A**) The EYFP-CENP-A K124 mutant is ubiquitylated and interacts with HJURP. In vivo ubiquitylation assay. CENP-A$^{-/F}$ RPE-1 cells were transfected with 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 ≥ 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). Please click here to view a larger version of this figure.

A



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 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]. Please click here to view a larger version of this figure.

B number	Relevant characteristic(s)	Reference	Source
B3027	pBabe-puro-Cre	Niikura et al., 2019	Dr. Amruta Ashtekar, Dr. Lawrence S. Kirschner
B3182	pBabe-EYFP	Niikura et al., 2019	-
B3161	pBabe-EYFP-CENP-A WT	Niikura et al., 2019	Dr. Daniele Fachinetti, Dr. Don W. Cleveland
B3164	pBabe-EYFP-CENP-A K124R	Niikura et al., 2019	Dr. Daniele Fachinetti, Dr. Don W. Cleveland
B3031	psPAX2	Niikura et al., 2019	Dr. John Thompson, Dr. Gustavo W. Leone
D3032	pMD2.g	Niikura et al., 2019	Dr. John Thompson, Dr. Gustavo W. Leone
B3189	pGP	Niikura et al., 2019	Dr. Kenji Tago (Jichi Medical Univeristy, Japan)
B3190	pCI-VSVG	Niikura et al., 2019	Dr. Kenji Tago (Jichi Medical Univeristy, Japan)
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	-

Table 1: Plasmid vectors used in this study.

B number	Helper/packaging plasmid vectors	Combination 1	Combination 2	Combination 3
B3031	psPAX2 (lentiviral gag/pol vector)	2mg		
D3032	pMD2.g (lentiviral env vector)	2mg		
B3189	pGP (retroviral gag/pol vector)		2mg	
B3190	pCI-VSVG (retroviral env vector)		2mg	
B3001	pPAM (amphotropic helper vector encoding retroviral gag-pol-env)			2mg

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# the development of lower molecular weight tagging or probing technique is urgently required to visualize ubiquitylation and/or to investigate spatiotemporal protein ubiquitylation signaling at the living cellular and the entire organism levels.

In the present study, EYFP vector control is essential for control assays and analyses (immunofluorescence analysis, colony outgrowth assay, and in vivo ubiquitylation assay) to discuss the outcome of major mass spectrometry analysis properly. Non-specific interaction with EYFP-protein is often observed in the immunoprecipitation experiment, thus EYFP vector control is indispensable to evaluate the true interaction of EYFP-fused protein(s). Our mass spectrometry analysis using EYFP-CENP-A K124R expressed in the RPE-1 CENP-A$^{-/F}$ cells did not show ubiquitylation on lysine sites in the EYFP polypeptide sequence. However, in other unknown conditions, it could be expected that the lysine site in the EYFP polypeptide sequence is ubiquitylated in EYFP- and/or other tagged fusion protein of high molecular weight.

For colony outgrowth assays, it is recommended to collect cells for western blot analysis to confirm protein depletion of endogenous CENP-A after retro-Cre virus infection and protein expression of pBabe-EYFP-CENP-A constructs. In this way, we can judge if the colony outgrowth phenotype is truly due to the sole expression of exogenous CENP-A WT or mutants. For any western blot analysis, if stripping is performed to reblot the membrane with different antibodies for the next turn of western blot analysis, one should use the same quantitative detection system as a previous turn's blot for the next turn's blot with different antibodies. One should make sure that the bands in the previous turn's blot are undetectable at the aimed region of the membrane in the next turn's blot with different antibodies. Or use the same quantitative detection system as previous turn's blot and check if the incubation with the mere secondary antibody used in the previous turn's blot does not lead to the detection of protein bands before starting the next turn's blot with different antibodies. For in vivo ubiquitylation assay, it is important to run bigger SDS-PAGE gel using the apparatus to run bigger SDS-PAGE gel (Gel electrophoresis apparatus I or II). Empirically, bigger SDS-PAGE gel would separate protein bands clearly and enhance the sensitivity of the detection of the faint bands that could have been poorly detected in the smaller gel (i.e., protein bands appear sharper in bigger gel).

It is extremely important to choose proper SDS-PAGE gel to map post-translational modifications (PTMs) of a specific protein thorough LC-MA/MS. The most widely used gel system for SDS-PAGE is the Laemmli system, which uses Tris-glycine gels comprising a stacking gel component and the resolving gel component. In this classical system, the pH and ionic strength of the buffers used in the stacking gel (Tris, pH 6.8) and resolving gel (Tris, pH 8.8) are different from the buffer used for running the gel (Tris, pH 8.3). Band distortion, loss of resolution, or artifact bands may be caused by the highly alkaline operating pH of the Laemmli system. The previous report described major causes of poor band resolution with the Laemmli system[16], including instability and short expiration period of the resolving gel due to hydrolysis of polyacrylamide at the high pH, chemical alterations of sample proteins, reoxidation of reduced disulfides of cysteine residues of proteins, and cleavage of Asp-Pro bonds

of proteins with heating at 95-100 °C in Laemmli sample buffer at pH 5.2. The commercially available 4%-12% Bis-Tris protein gels are Bis-Tris HCl–buffered (pH 6.4) 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.

Disclosures

The authors declare no competing interests.

Acknowledgments

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