Video Article

Protein Purification Technique that Allows Detection of Sumoylation and Ubiquitination of Budding Yeast Kinetochore Proteins Ndc10 and Ndc80

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Abstract

Post-translational Modifications (PTMs), such as phosphorylation, methylation, acetylation, ubiquitination, and sumoylation, regulate the cellular function of many proteins. PTMs of kinetochore proteins that associate with centromeric DNA mediate faithful chromosome segregation to maintain genome stability. Biochemical approaches such as mass spectrometry and western blot analysis are most commonly used for identification of PTMs. Here, a protein purification method is described that allows the detection of both sumoylation and ubiquitination of the kinetochore proteins, Ndc10 and Ndc80, in *Saccharomyces cerevisiae*. A strain that expresses polyhistidine-Flag-tagged Smt3 (HF-Smt3) and Myc-tagged Ndc10 or Ndc80 was constructed and used for our studies. For detection of sumoylation, we devised a protocol to affinity purify His-tagged sumoylated proteins by using nickel beads and used western blot analysis with anti-Myc antibody to detect sumoylated Ndc10 and Ndc80. For detection of ubiquitination, we devised a protocol for immunoprecipitation of Myc-tagged proteins and used western blot analysis with anti-Ub antibody to show that Ndc10 and Ndc80 are ubiquitinated. Our results show that epitope tagged-protein of interest in the His-Flag tagged Smt3 strain facilitates the detection of multiple PTMs. Future studies should allow exploitation of this technique to identify and characterize protein interactions that are dependent on a specific PTM.

Video Link

The video component of this article can be found at https://www.jove.com/video/52482/

Introduction

Ubiquitination and sumoylation allow the conjugation of ubiquitin and Small Ubiquitin-like MOdifier (SUMO; Smt3 in *S. cerevisiae* [1]) to a target protein, respectively. PTMs of kinetochore proteins affect their cellular levels and protein-protein interactions during different cell cycle phases to ensure faithful chromosome segregation. For example, cellular levels of Cse4/CENP-A and outer kinetochore protein Dsn1 are regulated by ubiquitin-mediated proteolysis for ensuring genome stability [2-5]. Destabilization of incorrect kinetochore-microtubule attachments requires the Ipl1/Aurora B kinase, which phosphorylates Dam1 and Ndc80 complexes that directly interact with microtubules [6-8]. Despite the identification of over seventy kinetochore proteins, there are very few studies that investigate the modifications of these proteins with PTMs, *e.g.*, ubiquitin and SUMO. A major limitation is the ability to preserve the PTMs during purification and the paucity of custom antibodies for detection of PTMs such as sumoylation, phosphorylation, methylation, and others. Characterization of sumoylated kinetochore proteins Ndc10, Cep3, Bir1, and Ndc80 utilized a custom antibody [9]. Additionally, Ndc10 has been implicated as a substrate for ubiquitination [10]. Human Hec1 (Ndc80 in *S. cerevisiae*) is also substrate for ubiquitination, regulated by APC/C-hCdh1 E3 ligase [11]. Therefore, Ndc10 and Ndc80 are good candidates for optimization of the protocol to detect both sumoylation and ubiquitination in *S. cerevisiae*.

To facilitate the identification of sumoylation, we constructed strains that express HF-Smt3 and Myc-tagged Ndc10 or Ndc80. The use of epitope tags (HF: His6-Flag) minimizes the background due to cross-reactivity that is frequently observed in polyclonal serum raised against a candidate protein. We devised a protocol to affinity purify HF-Smt3 conjugates and then used commercial anti-Flag and anti-Myc antibodies to detect the presence of sumolyated Ndc10 and Ndc80 in the purified Smt3 preparation. For ubiquitination, we devised a modified immunoprecipitation protocol that preserves ubiquitination of the Myc-tagged kinetochore proteins and performed western blot analysis with commercial anti-Ub antibody to detect ubiquitination of Ndc10 and Ndc80.

Protocol

1. Growth of Yeast Cells

1. Inoculate yeast cells in 30 ml of YPD (**Table 1**) in a small flask. Incubate at 30 °C overnight with shaking.
2. Dilute the cells to an optical density of 0.2 at 600 nm (OD_{600} = 0.2) in 50 ml of YPD and incubate at 30 °C with shaking.
3. Grow the culture to an optical density of 1.0 at 600 nm (OD_{600} = 1.0).

4. Centrifuge cells for 5 min at 2,000 x g and discard the supernatant.
5. Resuspend the cell pellet in 40 ml sterile water and centrifuge for 5 min at 2,000 x g to wash the cells. Discard the supernatant and store the cell pellet at -20 °C.

2. Extraction of Proteins

1. Resuspend cells in 0.5 ml of ice-cold guanidine buffer (Table 1) for pull-down assay using Ni-NTA superflow beads or buffer A (**Table 1**) for immunoprecipitation. Keep tubes on ice at all times.
2. Transfer to 2 ml screw cap tube.
3. Add the same volume of glass beads (Table of **Materials**).
4. Bead-beat the cells in a mini bead beater (Table of Materials) for 2 min at room temperature, then place on ice for 2-3 min. Repeat this three times.
5. Vortex on high speed at 4 °C for 30-60 min. Check the cells by visualization under the microscope to ensure that the cells are lysed.
   Note: Lysed cells will appear as dark ghosts and lack a boundary or defined shape. Optimally at least 80% of the cells should be lysed.
6. Puncture a hole in the bottom of the tube using a push pin and place in a collection 15 ml conical tube (screw cap should be loose).
7. Centrifuge at 1,000 x g for 1 min to collect the lysate.
8. Transfer the lysate to a micro centrifuge tube.
9. Centrifuge at 15,000 x g for 30 min at 4 °C to collect the extracted proteins.
10. Measure the concentration of the extracted proteins using the protein assay kit (Table of **Materials**) and normalize all extracts to contain the same amount of protein. Bring the total volume to 1 ml with appropriate buffer.
    Note: Around 5 mg of total protein is obtained from 50 OD_{600} cells.
11. Save 50 µl (250 µg protein if the total protein extracted from step 2.10 is 5 mg) as whole cell extract (WCE). Add 50 µl of 2x Laemmli sample buffer (**Table 1**) and incubate at 100 °C in a heat block for 3-5 min. Load 10 µl of each sample in a SDS-PAGE gel for Western Blot analysis (Section 5: Western blot analysis).

3. Purification of HF-Smt3 Conjugates

1. Obtain the Ni-NTA superflow beads (Table of **Materials**) necessary for the experiments (100 µl of beads per sample) by low speed centrifugation (800-1,500 x g) for 1 min. Remove the supernatant.
2. Wash beads 5x with 1 ml of PBS (Table of Materials): Invert top-over-bottom until the beads are resuspended, collect beads by low speed centrifugation, and remove the supernatant.
3. Suspend beads in 1 ml of guanidine buffer (**Table 1**) and aliquot them into the number of tubes corresponding to samples to be processed. Collect beads by low speed centrifugation, and remove the supernatant. Mix the beads well by inverting top-over bottom.
   Note: Mix beads well by inverting top-over bottom.
4. For each sample, add 950 µl of the remaining WCE (from Step 2.11: Extraction of proteins) to 100 µl of Ni-NTA superflow beads.
5. Incubate on a rocking platform at 4 °C for at least 4 hr or overnight.
6. Centrifuge at 800-1,500 x g for 1 min at 4 °C.
7. Save 50 µl as supernatant (SUP). Add 50 µl of 2x Laemmli sample buffer and incubate at 100 °C in a heat block for 3-5 min. Load 10 µl of each sample in an SDS-PAGE gel for Western Blot analysis (Section 5: Western blot analysis).
8. Wash beads once with 1 ml of guanidine buffer for 5-10 min on a rocking platform.
9. Wash beads 5x with 1 ml of breaking buffer (**Table 1**) for 5-10 min on a rocking platform.
10. Resuspend beads in 90 µl of 2x Laemmli sample buffer.
11. Add 10 µl of 1 M imidazole.
    Note: Imidazole helps to dissociate the His-tagged protein from the Ni-NTA beads due to competing interaction between imidazole and the beads.
12. Incubate at 100 °C in a heat block for 3-5 min.
13. Vortex, then centrifuge at 13,000 x g for 30 sec. Transfer the supernatant to a fresh tube.
14. Load 10-20 µl of each sample in an SDS-PAGE gel for Western Blot analysis (Section 5: Western blot analysis).
    Note: 10-20 µl corresponds to 0.5-1.0 mg of input, if 5 mg of WCE is used.

4. Immunoprecipitation of Myc-tagged Kinetochore Proteins

1. Obtain anti-c-Myc agarose affinity gel antibody (Table of **Materials**) necessary for the experiments (25 µl of resin per sample) by low speed centrifugation (800-1,500 x g). Remove the supernatant.
2. Wash resin 5x with 1 ml of buffer A: Invert top-over-bottom until the resin is resuspended, collect resin by low speed centrifugation, and remove the supernatant.
3. Suspend resin in 1 ml of buffer A and aliquot them into the number of tubes corresponding to samples to be processed. Collect resin by low speed centrifugation, and remove the supernatant.
   Note: Mix the resin well by inverting top-over-bottom.
4. Add 950 µl of the remaining WCE (from Step 2.11: Extraction of proteins) to 25 µl of resin.
5. Incubate on a rocking platform at 4 °C overnight.
6. Centrifuge at 800-1,500 x g for 1 min at 4 °C.
7. Save 50 µl as supernatant (SUP). Add 50 µl of 2x Laemmli sample buffer and incubate at 100 °C in a heat block for 3-5 min. Load 10 µl of each sample in an SDS-PAGE gel for Western Blot analysis (Section 5: Western blot analysis).
8. Wash resin 5x with 1 ml of buffer A: Invert top-over-bottom until the resin is resuspended, collect resin by low speed centrifugation, and remove the supernatant.
9. Resuspend the resin in 100 µl of SUMEB sample buffer (**Table 1**).
   Note: SUMEB sample buffer contains 8 M Urea and 1 % SDS (strong denaturing condition).

10. Incubate at 100 °C in a heat block for 3-5 min.
11. Vortex, then centrifuge at 13,000 x g for 30 sec. Transfer the supernatant to a fresh tube.
12. Load 10-20 µl of each sample in an SDS-PAGE gel for Western Blot analysis (Section 5: Western blot analysis).
    Note: 10-20 µl corresponds to 0.5-1.0 mg of input, if 5 mg of WCE is used.

5. Western Blot Analysis

1. Load the protein extracts on 4-12% Bis-Tris gels (Table of Materials) and perform electrophoresis at 120 V for 90 min (Running buffer: Table of **Materials**).
2. Transfer the proteins from gel to a nitrocellulose membrane (Table of **Materials**) using a transfer apparatus at 30 V for 90 min (Transfer buffer: Table of **Materials**).
3. Block the membrane in 5% milk/1x TBST (**Table1**) for 1 hr at room temperature.
4. Incubate membrane with primary antibody (Table of **Materials**) in 5% milk/1x TBST overnight at 4 °C. For primary antibodies, use a dilution of 1:1,000 (anti-Flag and anti-Ub antibodies) or 1:5,000 (anti-Myc antibody).
5. Wash membrane 3x for 10 min each with 1x TBST.
6. Incubate membrane with secondary antibody (Table of **Materials**) in 5% milk/1x TBST for 1 hr at room temperature. Use a 1:5,000 dilution of secondary antibodies.
7. Wash membrane 3x for 10 min each with 1x TBST.
8. Incubate membrane with ECL working solution (Table of **Materials**) for 5 min.
9. Expose the membrane to blue sensitive X-Ray film (Table of **Materials**) and develop using an automatic developer (Table of **Materials**).

Representative Results

To detect sumoylation of kinetochore proteins Ndc80 and Ndc10, strains with HF-Smt3 and Myc-tagged kinetochore proteins (Ndc80 or Ndc10) were constructed (**Table 2**), as previously described [9,12]. HF-Smt3 conjugates were affinity purified using Ni-NTA beads. Western blot analysis of purified HF-Smt3 with an anti-Flag antibody allowed detection of sumoylated forms of SUMO substrates that were absent in the control strain without HF-Smt3 (**Figure 1A** and **1B**, left panel). As expected, multiple forms of SUMO substrates were detected in the HF-Smt3 strain. We next determined if Ndc80 and Ndc10 are present in the purified HF-Smt3 conjugate by doing western blot analysis using an anti-Myc antibody. Multiple bands that were of higher molecular weight than that of Ndc80 or Ndc10 were clearly detected (**Figure 1A** and **1B**, right panel). Moreover, multiple bands of both Ndc80 and Ndc10 were reduced in nocodazole treated cells (**Figure 1C** and **1D**). These results show that protein purification and western blot analysis described here allow the detection of sumoylation of Ndc80 and Ndc10, as previously described[9].

To detect ubiquitination of Ndc80 and Ndc10, Myc-tagged Ndc80 or Ndc10 was immunoprecipitated (IP) and western blot analysis was performed with anti-Myc and anti-Ub antibodies (**Figure 2**). Analysis of whole cell extracts (WCE) and supernatant (SUP) confirmed the expression of Ndc80-Myc and Ndc10-Myc (**Figure 2A**). The lower molecular weight bands on the WCE may represent degradation products. IP samples probed with anti-Myc showed multiple high molecular weight bands, suggesting that Ndc80 and Ndc10 contain PTMs. A laddering pattern of IP samples probed with anti-Ub antibody showed that Ndc80 and Ndc10 are ubiquitinated (**Figure 2A**, α-Ub). The laddering pattern of Ndc80 and Ndc10 were enhanced by treatment with proteasome inhibitor (MG132) (**Figure 2B**, α-Ub), further confirming that these bands represent poly-ubiqutination. The representative results reveal that both Ndc80 and Ndc10 are substrates for sumoylation and ubiquitination in *S. cerevisiae* and that the described protein purification methods are useful for detection of PTMs such as sumoylation and ubiquitination. According to our knowledge, this is the first report that Ndc80 in *S. cerevisiae* is a substrate for ubiquitination, although ubiquitin mediated proteolysis of the human homolog Hec1 has been previously published [11].

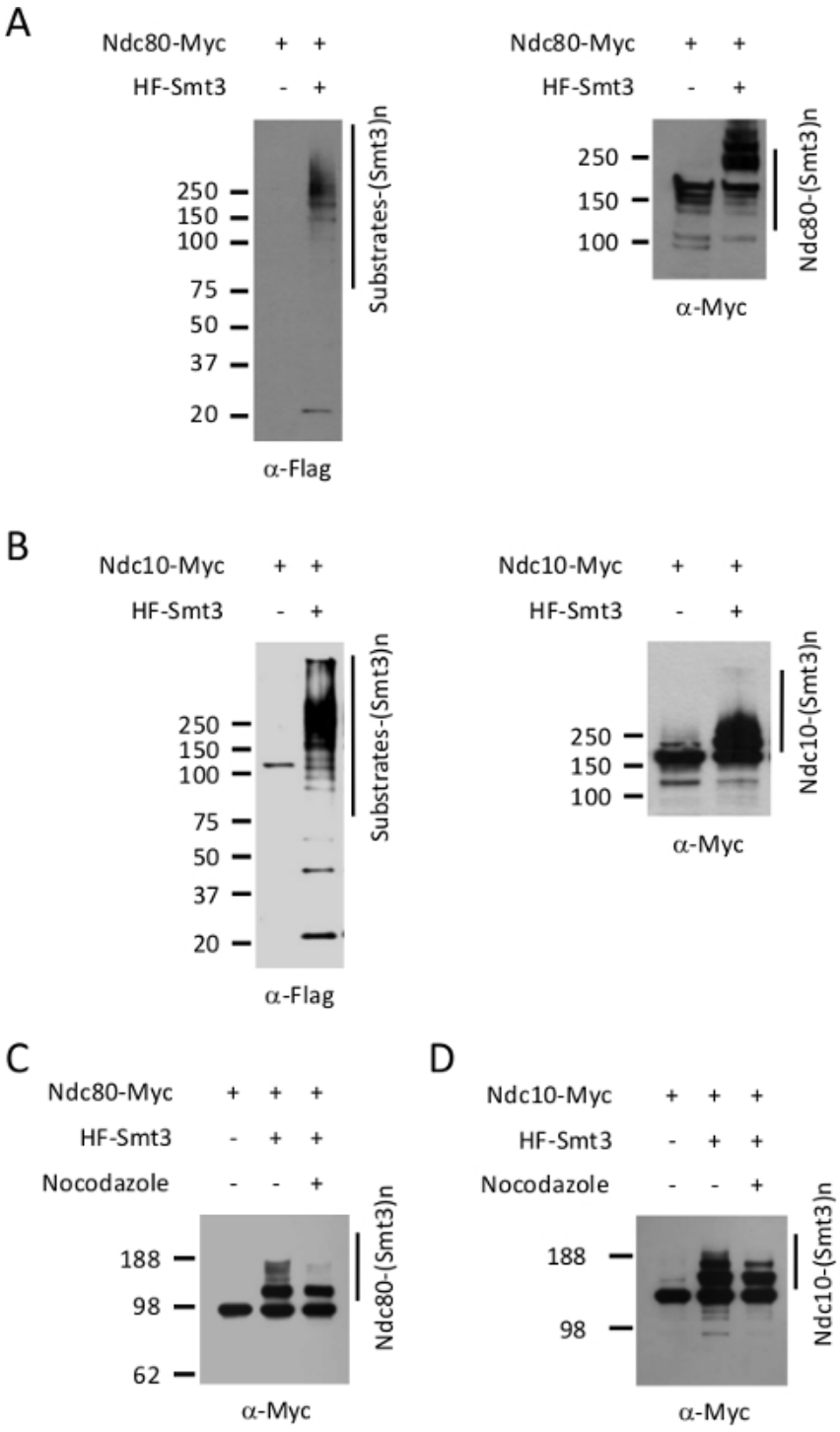

Figure 1: Purification of sumolyated substrates allows the identification of kinetochore proteins Ndc80 and Ndc10 as SUMO substrates. Proteins were extracted and HF-Smt3 conjugates were prepared and analyzed by western blot analysis after SDS-PAGE separation. (**A** and **B**) Ndc80 and Ndc10 are sumoylated. Proteins were extracted from logarithmically growing cells in YPD. Left panel: Total SUMO substrates were detected with an anti-Flag antibody. Right panel: Sumoylated Ndc80 or Ndc10 was detected in the HF-Smt3 conjugates when probed with an anti-Myc antibody. (**C** and **D**) Sumoylation of Ndc80 and Ndc10 is reduced in nocodazole treated cells. Logarithmically growing cells in YPD were treated with DMSO (-) or DMSO + 20 µg/ml nocodazole (+) for 2 hr at 30 °C. HF-Smt3 conjugates were analyzed by western blot analysis using anti-Myc antibody. Isogenic yeast strains used are (**A** and **C**) YPH1800 (Ndc80-Myc), YMB7862 (His-Flag-SMT3 Ndc80-Myc) and (**B** and **D**) YPH1734 (Ndc10-Myc), YMB7867 (His-Flag-SMT3 Ndc10-Myc).

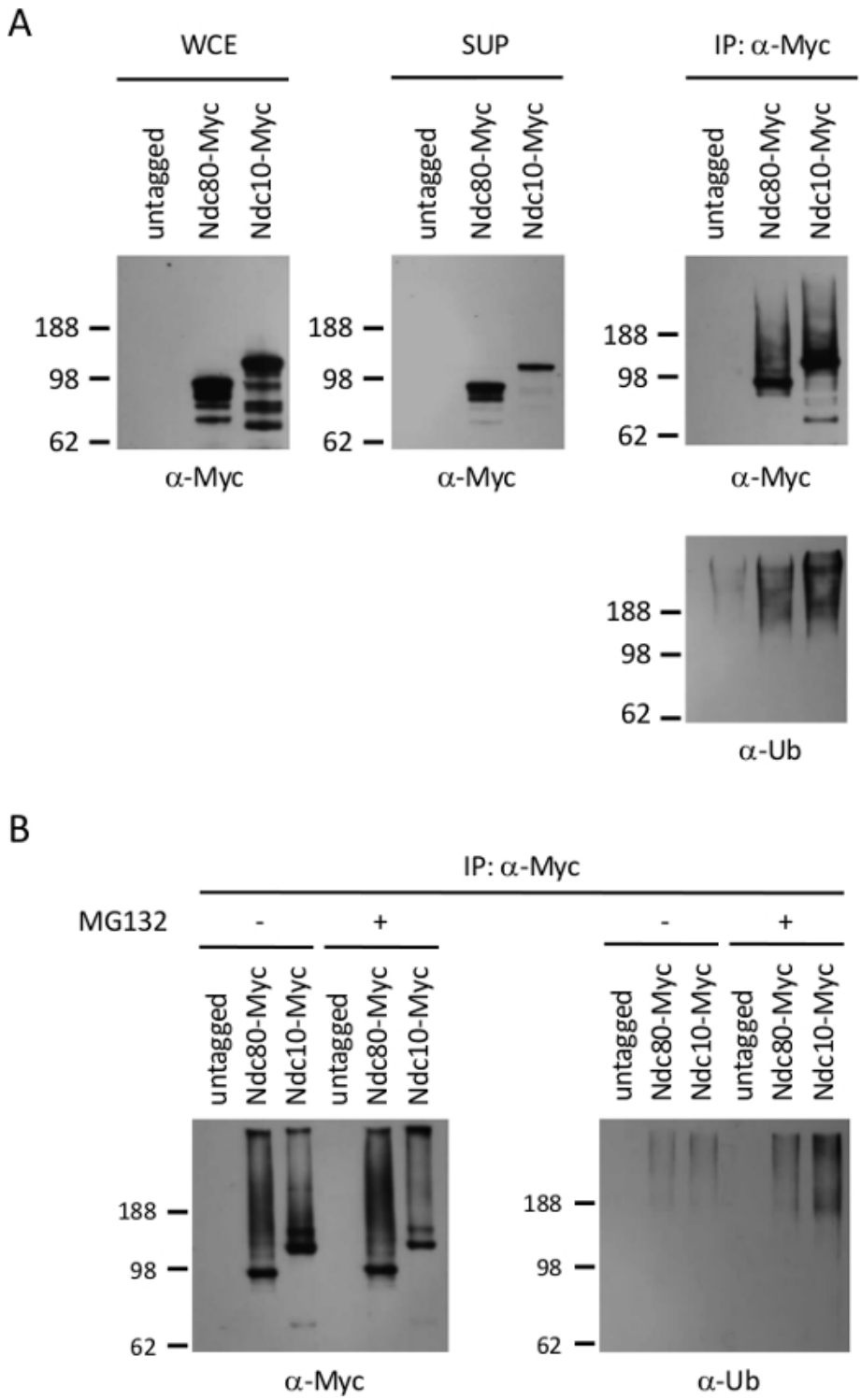

Figure 2: Ubiquitination of kinetochore proteins Ndc80 and Ndc10. Extraction of protein and immunoprecipitation were performed as described in the protocol. (**A**) Ndc80 and Ndc10 are ubiquitinated. Proteins were extracted from logarithmically growing cells in YPD. Whole cell extract (WCE), supernatant (SUP), and the immunoprecipitated (IP) fractions were subjected to SDS-PAGE. Western blots were used to detect Myc-tagged and ubiquitinated proteins with anti-Myc and anti-Ub antibodies, respectively. (**B**) Ubiquitination of Ndc80 and Ndc10 is enhanced in cells treated with proteasome inhibitor MG132. Logarithmically growing cells in YPD were treated with DMSO (-) or DMSO + 50 μM MG132 (+) in the presence of 0.003% of SDS for 3 hr at 30 °C, as previously described [13]. Western blots of IP fraction were done using anti-Myc or anti-Ub antibody. Isogenic yeast strains used are YMB7278 (His-Flag-SMT3), YMB7862 (His-Flag-SMT3 Ndc80-Myc) and YMB7867 (His-Flag-SMT3 Ndc10-Myc).

Solution	Components

YPD	1 % yeast extract, 2 % bacto-peptone, 2 % glucose
Guanidine buffer	0.1 M Tris-HCl pH 8.0, 6 M guanidine chloride, 0.5 M NaCl
Buffer A	50 mM Tris-HCl pH 7.5, 50 mM NaCl, 0.2 % Triton X-100, 1x protease inhibitors
Breaking buffer	0.1 M Tris-HCl pH 8.0, 20 % glycerol, 1 mM PMSF
SUMEB sample buffer	1 % SDS, 8 M Urea, 10 mM MOPS pH 6.8, 10 mM EDTA, 0.01 % bromophenol blue
2x Laemmli Sample Buffer	100 mM Tris-HCl pH 6.8, 4 % SDS, 20 % glycerol, 0.2 % bromophenol blue (Before use: add b-mercaptoethanol (BME) to a final concentration of 200 mM)
1x TBST	137 mM Sodium Chloride, 20 mM Tris-Hcl pH 7.5, 0.1% Tween-20

Table 1: Solutions.

Table 2. Yeast strains used in this study*.			
Strains	Parent	Genotype	Reference
BY4741		MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0	Open Biosystems
BY4742		MATa his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0	Open Biosystems
YMB7278	BY4741/BY4742	MATa his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 HF-SMT3::LEU2	This study
YPH1734		MATa ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 NDC10-13Myc::kanMX6	Montpetit *et al*., 2006
YPH1800		MATa ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 NDC80-13Myc::His3MX6	Montpetit *et al*., 2006
YMB7862	YMB7278/YPH1800	MATa his3 leu2 ura3 ade2 trp1 HF-SMT3::LEU2 NDC80-Myc::His3MX6	This study
YMB7867	YMB7278/YPH1734	MATa his3 leu2 ura3 trp1 lys2 HF-SMT3::LEU2 NDC10-Myc::kanMX6	This study
* All yeast strains are derived from Saccharomyces cerevisiae S288C.			

Table 2: Yeast strains used in this study.

Discussion

Epitope tags such as HA, Myc, Flag, and GST are widely used for biochemical analysis of proteins. Construction of strains with HF-Smt3 and Myc-tagged kinetochore proteins, such as Ndc10 and Ndc80, facilitates the detection of PTMs such as sumoylation and ubiquitination. HF-Smt3 pull down assay allows the detection of sumoylated kinetochore proteins, Ndc10 and Ndc80 (**Figure 1**). The affinity purification protocol and western blot analysis using anti-Flag antibody establish the specificity of interaction between HF-Smt3 and target proteins, as modified proteins were not detected in the control strain without HF-Smt3. The use of the Myc tag on the protein substrates validates the presence of sumoylated Ndc10 and Ndc80 in the HF-Smt3 conjugates. Furthermore, sumoylation of both Ndc10 and Ndc80 was reduced in nocodazole treated cells (**Figure 1C** and **1D**). Decreased sumoylation of Ndc10 in response to nocodazole treatment, but not Ndc80, was previously reported by Montpetit *et al*. [9]. This may be due to differences in experimental protocol and use of a custom anti-SUMO antibody. Our results for sumoylation of kinetochore substrates suggest that a similar protocol can be used to investigate other candidate SUMO substrates.

To detect ubiquitination, Myc-tagged kinetochore proteins were first immunoprecipitated and the IP fractions were probed with anti-Ub antibody (**Figure 2**). The laddering pattern of Ndc10 and Ndc80 was increased when cells were treated with MG132 to inhibit proteasome function (**Figure 2B**), indicating that these proteins are substrates of the proteasome. The IP fractions failed to detect sumoylation of Ndc10 or Ndc80 in western blot analysis with anti-Flag or anti-Smt3 antibody (data not shown). This may be due to technical reasons such as levels of sumoylated substrate in the IP fraction or the interference from the epitope tags. Further optimization of the IP protocol such as concentration of salts and western blotting conditions should facilitate the detection of multiple PTMs of any protein of interest. At present, sumoylation is best detected by the HF-Smt3 pull down assay followed by western blots of the candidate SUMO substrates (**Figure 1**).

Several details affect the efficiency of protein purification and the ability to detect the PTMs. First, all tubes should be kept on ice to inhibit the proteases except as specified. Second, a 1:1 ratio of cell suspension to glass beads is critical to disrupt the cells by the bead beater and/or

vortexer. Cell lysis should be monitored by examination of the cells under the microscope. Third, it is very important to prepare a clarified lysate for the pull down assay or IP. Short or low speed centrifugation can contribute to contamination of cell debris in the lysate and this affects the ability to detect the PTMs. Finally, vigorous shaking of the beads with a large volume of clear lysate (about 1 ml) in a 1.5 ml micro centrifuge tube ensures that the beads are fully suspended for the pull down assay or IPs. In summary, protein purification and detection techniques such as that described here provide important mechanistic insights into how PTM of kinetochore proteins affect their structure and assembly for faithful chromosome segregation [14,15].

Disclosures

The authors have nothing to disclose.

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