



Title	A novel method for purification of the endogenously expressed fission yeast Set2 complex
Author(s)	Suzuki, Shota; Nagao, Koji; Obuse, Chikashi; Murakami, Yota; Takahata, Shinya
Citation	Protein Expression and Purification, 97, 44-49 <a href="https://doi.org/10.1016/j.pep.2014.02.005">https://doi.org/10.1016/j.pep.2014.02.005</a>
Issue Date	2014-05
Doc URL	<a href="http://hdl.handle.net/2115/56352">http://hdl.handle.net/2115/56352</a>
Type	article (author version)
File Information	PEP_97_44-.pdf



[Instructions for use](#)

## **A Novel Method for Purification of the Endogenously Expressed Fission Yeast Set2 Complex**

Shota Suzuki<sup>a</sup>, Koji Nagao<sup>b</sup>, Chikashi Obuse<sup>b</sup>, Yota Murakami<sup>c</sup>, and Shinya Takahata<sup>c\*</sup>

<sup>a</sup>Graduate School of Chemical Science and Engineering, Hokkaido University, Japan

<sup>b</sup>Graduate School of Life Science, Hokkaido University, Japan

<sup>c</sup>Department of Chemistry, Faculty of Science, Hokkaido University, Japan

\*Corresponding author: Shinya Takahata. Bioorganic Chemistry Laboratory, Department of Chemistry, Faculty of Science, Hokkaido University, N10 W8 Kita-Ku, Sapporo City, Hokkaido, Japan 060-0810.

Tel: +81-(0)11-706-3815; Fax: +81-(0)11-706-4924; E-mail:

[shinya.takahata@mail.sci.hokudai.ac.jp](mailto:shinya.takahata@mail.sci.hokudai.ac.jp)

## Abstract

Chromatin-associated proteins are heterogeneously and dynamically composed. To gain a complete understanding of DNA packaging and basic nuclear functions, it is important to generate a comprehensive inventory of these proteins. However, biochemical purification of chromatin-associated proteins is difficult and is accompanied by concerns over complex stability, protein solubility and yield. Here, we describe a new method for optimized purification of the endogenously expressed fission yeast Set2 complex, histone H3K36 methyltransferase. Using the standard centrifugation procedure for purification, approximately half of the Set2 protein separated into the insoluble chromatin pellet fraction, making it impossible to recover the large amounts of soluble Set2. To overcome this poor recovery, we developed a novel protein purification technique termed the filtration/immunoaffinity purification/mass spectrometry (FIM) method, which eliminates the need for centrifugation. Using the FIM method, in which whole cell lysates were filtered consecutively through eight different pore sizes (53–0.8  $\mu\text{m}$ ), a high yield of soluble FLAG-tagged Set2 was obtained from fission yeast. The technique was suitable for affinity purification and produced a low background. A mass spectrometry analysis of anti-FLAG immunoprecipitated proteins revealed that Rpb1, Rpb2 and Rpb3, which have all been reported previously as components of the budding yeast Set2 complex, were isolated from fission yeast using the FIM method. In addition, other subunits of RNA polymerase II and its phosphatase were also identified. In conclusion, the FIM method is valid for the efficient purification of protein complexes that separate into the insoluble chromatin pellet fraction during centrifugation.

**Highlights:**

- A novel filtration method for yeast clear lysate preparation is proposed.
- Fission yeast Set2-associating factors were identified using this method.
- The filtration method may be useful for purification of other chromatin factors.

**Key words:** Chromatin; Set2; H3K36; SRI domain; RNA polymerase II; FIM.

**Abbreviations:** CTD, carboxyl terminal domain; FIM, filtration/ immunoaffinity purification/ mass spectrometry; H3K36, histone H3 lysine 36; HDAC, histone deacetyltransferase complex; SRI, Set2-Rpb1 interaction.

## Introduction

Yeast systems are useful for studying the genetics of chromatin factors because genomic modifications and analysis of mutants are relatively easy. Chromatin is comprised of DNA and histone proteins, the latter of which are regulated at the post-transcriptional level by histone-modifying enzymes. Numerous histone-modifying enzymes have been reported to date, and the methylation status of lysine residues at the N-terminal tail of histone H3 determines the transcriptional activity of chromatin [1]. The Set2 methyltransferase protein, which contains a SET domain that methylates lysine 36 of histone H3 (H3K36), plays an important role in transcriptional elongation [2]. Methylation of H3K36 is conserved from yeast to human and is required for recruitment of the Rpd3S histone deacetyltransferase complex (HDAC), which represses cryptic transcription from internal transcription start sites [3, 4]. In addition, an anti-silencing effect of H3K36 methylation that occurs independently of Rpd3S HDAC has also been reported, suggesting an additional, as yet uncharacterized, role of H3K36 methylation [5]. Furthermore, differential effects of histone H2B mono-ubiquitylation and deubiquitylation, H3K4 methylation, and H3K36 methylation on transcription have been reported, suggesting the existence of a complicated interplay between histone-modifying enzymes [6].

*In vitro*, Set2 from budding yeast associates with the Ser-2 and Ser-5 phosphorylated carboxyl terminal domain (CTD) of Rpb1, a core component of RNA polymerase II that contains multiple repeats of the consensus heptamer “YSPTSPS”, via its Set2-Rpb1 interaction (SRI) domain [7]. Deletion of the SRI domain in Set2 causes defects in H3K36 methylation *in vivo* [8], suggesting that its association with RNA polymerase II is required for this histone modification. The SET and SRI domains of Set2 are highly conserved between budding yeast and fission yeast, while conservation of other regions of the protein is very poor. However, in contrast to the budding yeast Set2 complex, only a few studies of fission yeast Set2 have been performed to date [9, 10].

Recent studies have developed optimized techniques for purifying protein complexes, making it possible to isolate weakly associated proteins [11, 12]. Despite these advancements, purification of chromosome-associated proteins, which migrate into the insoluble pellet during whole cell extract preparation, is still challenging. In some cases, DNase and/or RNase treatment can help to solubilize these proteins, but the majority of chromatin factors still remain in the pellet after treatment. Although single step immunoaffinity purification of the fission yeast Set2 complex using a standard centrifugation procedure to separate the soluble and insoluble fractions (Fig. 1) is typically successful, the yield is usually low and the procedure requires large amounts of cells and

antibodies. Furthermore, approximately half of the Set2 protein is lost in the chromatin pellet during the centrifugation step required to separate the clear lysate, cell debris and lipids. High salt concentrations, the addition of detergents, or denaturation of the chromatin pellet are effective strategies for solubilization of proteins [13, 14]; however, none of these procedures were useful for purifying fission yeast Set2. Similarly, although chromatin shearing by nuclease treatment is an effective technique for purification of core histones [15], soluble Set2 could not be obtained enough using this method.

The aim of this study was to develop an improved system to overcome the low yield of purified fission yeast Set2 obtained using the standard centrifugation procedure. We developed a novel technique for the preparation of a clear lysate, termed the filtration/immunoaffinity purification/mass spectrometry (FIM) method, in which the centrifugation step is replaced by consecutive filtration of the sample through descending pore sizes. Here, we describe the details of this technique and compare the results of a mass spectrometry analysis of the purified Set2 complex from fission yeast with those reported previously for purified budding yeast Set2. The results presented suggest that the FIM method is able to capture proteins that associate weakly with Set2.

## **Materials and methods**

### *Strains and media*

The fission yeast strains used in this study were FY2002 (*h+ ade6-DN/N leu1-32 ura4-DS/E imr1L::ura4<sup>+</sup> otr1R::ade6<sup>+</sup>*) [16] and SS259 (*h+ ade6-DN/N leu1-32 ura4-DS/E imr1L::ura4<sup>+</sup> otr1R::ade6<sup>+</sup> set2<sup>+</sup>::FLAG(3)::KanMX*). For 3× FLAG-tagging of the carboxyl terminus of Set2, the pFA6a-6xGly-3xFLAG-KanMX4 plasmid [17] was used to amplify the tagging cassette by polymerase chain reaction (PCR). All strains were grown in YES media at 30°C to a final density of  $1 \times 10^7$  cells/ml.

### *Whole cell extract preparation*

Cells were cultured in 4 L of YES media, harvested by centrifugation, washed once with ice-cold phosphate buffered saline (PBS), and then resuspended in 140 ml of 0.3 Buffer T (25 mM HEPES-KOH (pH 7.5), 0.1 mM EDTA, 10% glycerol, 0.3 M KCl, and 0.1% Tween 20) containing 1 mM phenylmethylsulfonyl fluoride and protease inhibitor cocktail (0.35 µg/ml benzamidine, 0.7 µg/ml pepstatin, and 0.5 µg/ml leupeptin). The cells were mixed with pre-chilled glass beads (0.5 mm diameter) and lysed using a BeadBeater (Biospec Products) for 60 s at 4°C. The lysis procedure was performed 20 times, resulting in

breakage of 60–70% of the cells. The whole cell extracts were incubated for 4 h at 4°C with 300 U of DNase I, 5 µl of RNase A (10 mg/ml), 8 mM DTT, and 5 mM MgCl<sub>2</sub>. Consecutive filtration of the extracts through the following descending pore sizes was performed using a vacuum filter system (Nalgene): 53 µm (Sanpo), 25 µm (Sanpo), 20 µm (Whatman 41), 9 µm (Advantec 5A), 5 µm (Advantec 3), 3 µm (Advantec 6), 1 µm (Advantec 4A), and a 0.8 µm nitrocellulose membrane (Millipore). The filtration procedure was completed in approximately 90 min. The filtrated lysates were used for immunoblotting and immunoprecipitation. For whole cell extracts prepared using the standard centrifugation method, the cell lysates were treated with DNase I and RNase A as described above, and then centrifuged at 39,000 × g for 90 min at 4°C.

#### *Immunoaffinity purification of Set2-FLAG*

Before use, 500 µl of Dynabeads Pan Mouse IgG (Invitrogen) were equilibrated in 5 ml of PBS containing 1 mg/ml BSA overnight. The beads were then incubated with 10 µg of anti-FLAG M2 monoclonal antibody (Sigma) for several hours at 4°C. After washing with 0.3 Buffer T, the beads were incubated with 100 ml of whole cell extract at 4°C for 2 h to immunoprecipitate Set2-FLAG. The beads were then washed six times with 25 ml of 0.3 Buffer T and the immunoprecipitated proteins were eluted in 300 µl of 0.3 Buffer T containing 300 µg of 3× FLAG peptide.

#### *Silver staining*

After separation through a 5–20% gradient SDS-PAGE gel (SuperSep Ace; Wako), the protein bands were stained using the SilverQuest Silver Staining Kit (Invitrogen) and the basic method recommended by the manufacturer.

#### *Immunoblotting*

Membranes were probed with an anti-FLAG M2 monoclonal antibody (Sigma) or an anti-H3 CT rabbit polyclonal antibody (Millipore) at 4°C for 6h. Horseradish peroxidase-conjugated anti-mouse IgG or anti-rabbit IgG (GE Healthcare Life Science) were used as secondary antibodies and chemical luminescence was detected using the LAS digital imaging system (Fuji Film). The results of immunoblotting were quantified as absorbance units using MultiGauge version 2.3 software (Fujifilm).

#### *Liquid chromatography-tandem mass spectrometry*

Mass spectrometry was performed as described previously [18]. Briefly, the purified Set2 complex was separated by SDS-PAGE and, after trypsinization of the sliced gel, the protein was analyzed by liquid chromatography-tandem mass spectrometry. The raw data files were analyzed using Mascot software (Matrix Science) and the resulting sequences were used to search the *Schizosaccharomyces pombe* protein database.

## Results

### *Preparation of insoluble pellet-associated chromatin factors using consecutive filtrations*

Set2 is a chromatin factor that associates with the phosphorylated CTD of RNA polymerase II and methylates H3K36 located at the 3' region of transcribed genes; however, its biological role for the transcriptional regulation is still obscure [19–21]. In the initial experiment, Set2, RNA polymerase II and novel Set2-associated factors were purified from SS259 fission yeast expressing FLAG-tagged Set2 using the standard purification procedure [21]. In this experiment, yeast cells were disrupted using glass beads and the crude extracts were treated with DNase and RNase to shear the chromatin. After nuclease treatment, the cell debris and supernatant were separated by centrifugation (Fig. 1). Immunoblotting of the pellet and supernatant fractions revealed that almost half of the FLAG-tagged Set2 remained in the pellet after the nuclease treatment (Fig. 2A). With the aim of achieving a more efficient recovery of Set2, we developed a consecutive filtration method in which crude extracts were applied to eight filters with descending pore sizes to eliminate cell debris without the need for centrifugation (Fig. 1). The final filter (0.8  $\mu\text{m}$ ) was easily clogged by debris; therefore, purification of the lysates through seven consecutively larger pore size filters was used to reduce the sample preparation time to approximately 90 min. The amount of FLAG-tagged Set2 in the supernatant prepared using the filtration method was markedly higher than that in the supernatant prepared using the centrifugation method (Fig. 2A). Furthermore, immunoblotting revealed that approximately equal amounts of Set2 and H3 were present in the filtered supernatant (Fig. 2A). To evaluate the efficiency of Set2 recovery using the two techniques, the total lysate, filtrated lysate, centrifuged supernatant, and centrifuged pellet were serially diluted and analyzed by quantitative immunoblotting. The amount of Set2 protein in the centrifuged supernatant was approximately 40% of that in the total lysate, while approximately 70% of the total Set2 protein remained in the filtrated lysate (Fig. 2B). Because detection of weakly associated factors by mass spectrometry requires efficient recovery of large amounts of target proteins, these results suggest that the



centrifugation method is unsuitable for preparation of the Set2 complex from fission yeast whole cell extracts.

We then determined the loss of Set2 protein at each of the eight filtration steps. Samples of the lysate (5  $\mu$ l) were collected after each filtration and the amount of Set2 protein present in each sample was determined by immunoblotting. After passing through the 5  $\mu$ m pore size filter, approximately 30% of Set2 was lost (Fig. 3). This loss may be attributable to the removal of unbroken yeast cells, which are typically 3–4  $\mu$ m in diameter and 7–14  $\mu$ m in length and would therefore fail to pass the 5  $\mu$ m pore size filter.

#### *Identification of Set2-interacting proteins*

Next, we compared the efficiencies of immunoprecipitation of Set2 from the filtrated lysate and the centrifuged supernatant. In this experiment, Set2 was immunoprecipitated using an anti-FLAG monoclonal antibody and the Set2-FLAG complex was released from the anti-FLAG antibody by the addition of a thirty-fold excess of 3 $\times$  FLAG peptides. The amount of Set2 immunoprecipitated from the centrifuged supernatant was 30–40% lower than that immunoprecipitated from the filtrated lysate, which reflected a similar difference in the input fraction (Fig. 4A). An immunoblot analysis of the immunoprecipitated filtered lysate using an anti-FLAG antibody revealed that full-length Set2-FLAG was recovered as the major band, although some cleaved products were also obtained (Fig. 4B). A number of Set2-associated proteins were also identified by silver staining of the immunoprecipitated filtered lysate on an SDS-PAGE gel (Fig. 4C). Affinity purification of a wild-type fission yeast strain (FY2002) using the anti-FLAG antibody produced a low background (Fig. 4C). A mass spectrometry analysis revealed that the Set2-FLAG co-immunoprecipitated proteins included Rpb1–5, Rpb7, and Rtr1 (Table 1).

## **Discussion**

#### *Removal of lipids from whole cell extracts using paper filters*

Chromatin comprises DNA and multiple proteins, and its relative density is high. After centrifuging whole cell extracts, chromatin complexes form a solid pellet, making it difficult to purify chromatin-associated proteins such as Set2 using the standard centrifugation procedure. In addition, the large quantities of lipids accrued from broken cells during cell lysis are a further hindrance to protein purification. Although high-speed centrifugation separates the contaminating lipids and produces a clear supernatant, chromatin-associated proteins separate into the insoluble pellet (Fig. 2), making them difficult to recover. To

overcome this problem, we developed a new FIM method to prepare whole cell lysates of fission yeast using consecutive filtering with conventional cellulose filter papers, thereby eliminating the need for a centrifugation step. Filter paper is useful for absorbing lipids in biochemical experiments [22] and the lipid absorbing feature of cellulose is utilized practically in the clinical setting [23]. Therefore, crude lysates were paper-filtrated to remove lipids and cell debris. Clear lysates were prepared from wild-type and Set2-FLAG(3)-expressing yeast strains using the filtration method; subsequent anti-FLAG immunoaffinity purification indicated a low background affinity and efficient recovery of Set2 and its associated factors without the need for centrifugation.

#### *Evaluation of the immunoaffinity-purified Set2 complex*

The Set2-associated proteins identified here included Rpb1–5, Rpb7, and Rtr1. Rpb1 and Rpb2 are the largest and second largest subunits of RNA polymerase II, respectively. These proteins have been identified previously as Set2-associated proteins [19] and, in a study of budding yeast, Rpb1 and Rpb2 were substoichiometrically co-purified with TAP-tagged Set2 [20]. Furthermore, Rpb1 and Rpb2 were isolated by Set2-FLAG immunoaffinity purification and 3× FLAG peptide elution of *Saccharomyces cerevisiae* whole cell lysates [21]. Rpb3 was also co-purified with Set2 [19].

Rpb4, Rpb5, Rpb7, and Rtr1 were identified here as novel Set2-associating factors. Rpb4 and Rpb7 form a heterodimer and function as a dissociable submodule of RNA polymerase II [24]. The Rpb4/7 heterodimer binds to ssDNA and ssRNA, and mediates a post-recruitment step in transcription initiation [25]. Co-purification of Set2 and Rpb4/7 suggests functional relationships between H3K36 methylation and dynamic changes in the architecture of the RNA polymerase II complex. Rpb5 binds directly to Rpb1, forming a jaw with Rpb9 that appears to grip the DNA for transcriptional initiation [26]. Notably, Rpb5 is a common subunit of RNA polymerases I, II and III [27]. Rtr1 is an Rpb1 CTD phosphatase that is responsible for the transition from Ser-5 phosphorylation to Ser-2 phosphorylation during transcriptional elongation [28]. Set2 binds preferentially to the Ser-2- and Ser-5-phosphorylated state of the Rpb1 CTD *in vitro* [7]. Therefore, Rtr1 may control the interaction between Set2 and Rpb1 *in vivo* via regulation of Rpb1 CTD phosphorylation. CTD phosphorylation is dynamically regulated; for example Rpb1 phosphorylated at both Ser-2 and Ser-5 is found only at the 3' sides of genes. Phosphorylation of Rpb1 may alter the stoichiometry or the stability of the RNA polymerase II complex and its interacting proteins. Set2 seems to associate with a particular state of the RNA polymerase II complex, which may explain why Rpb4, Rpb5, Rpb7 and Rtr1 were identified here as relatively low peptide

counts in the affinity-purified sample, and why other RNA polymerase II subunits were not detected by mass spectrometry. Further investigations are required to gain a more complete understanding of the mechanism of Set2 loading to the Rpb1 CTD during transcriptional elongation *in vivo*.

## **CONCLUSION**

The FIM method, which includes a combination of consecutive filtrations, immunoaffinity purification and mass spectrometry, is useful for purification of the Set2 complex purification and may be beneficial to the purification of other chromatin-associated factors.

## **ACKNOWLEDGMENTS**

We thank Aki Nakamura for technical assistance with the mass spectrometry analysis. This work was supported in part by the Global COE Program (Project No. B01: Catalysis as the Basis for Innovation in Materials Science) and by a Grant-in-Aid for Young Scientists from the Ministry of Education, Culture, Sports, Science and Technology in Japan.

## REFERENCES

- [1] T. Kouzarides, Chromatin modifications and their function. *Cell* 128 (2007) 693-705.
- [2] J.S. Lee, A. Shilatifard, A site to remember: H3K36 methylation a mark for histone deacetylation. *Mutat Res* 618 (2007) 130-134.
- [3] B. Li, M. Gogol, M. Carey, D. Lee, C. Seidel, J.L. Workman, Combined action of PHD and chromo domains directs the Rpd3S HDAC to transcribed chromatin. *Science* 316 (2007) 1050-1054.
- [4] E.J. Wagner, P.B. Carpenter, Understanding the language of Lys36 methylation at histone H3. *Nat Rev Mol Cell Biol* 13 (2012) 115-126.
- [5] R. Tompa, H.D. Madhani, Histone H3 lysine 36 methylation antagonizes silencing in *Saccharomyces cerevisiae* independently of the Rpd3S histone deacetylase complex. *Genetics* 175 (2007) 585-593.
- [6] Y. Zhang, Transcriptional regulation by histone ubiquitination and deubiquitination. *Genes Dev* 17 (2003) 2733-2740.
- [7] M. Li, H.P. Phatnani, Z. Guan, H. Sage, A.L. Greenleaf, P. Zhou, Solution structure of the Set2-Rpb1 interacting domain of human Set2 and its interaction with the hyperphosphorylated C-terminal domain of Rpb1. *Proc Natl Acad Sci U S A* 102 (2005) 17636-17641.
- [8] K.O. Kizer, H.P. Phatnani, Y. Shibata, H. Hall, A.L. Greenleaf, B.D. Strahl, A novel domain in Set2 mediates RNA polymerase II interaction and couples histone H3 K36 methylation with transcript elongation. *Mol Cell Biol* 25 (2005) 3305-3316.
- [9] S.A. Morris, Y. Shibata, K. Noma, Y. Tsukamoto, E. Warren, B. Temple, S.I. Grewal, B.D. Strahl, Histone H3 K36 methylation is associated with transcription elongation in *Schizosaccharomyces pombe*. *Eukaryot Cell* 4 (2005) 1446-1454.
- [10] K. Noma, S.I. Grewal, Histone H3 lysine 4 methylation is mediated by Set1 and promotes maintenance of active chromatin states in fission yeast. *Proc Natl Acad Sci U S A* 99 Suppl 4 (2002) 16438-16445.
- [11] O. Puig, F. Caspary, G. Rigaut, B. Rutz, E. Bouveret, E. Bragado-Nilsson, M. Wilm, B. Seraphin, The tandem affinity purification (TAP) method: a general procedure of protein complex purification. *Methods* 24 (2001) 218-229.
- [12] Y. Nakatani, V. Ogryzko, Immunoaffinity purification of mammalian protein complexes. *Methods Enzymol* 370 (2003) 430-444.

- [13] L. Vuillard, C. Braun-Breton, T. Rabilloud, Non-detergent sulphobetaines: a new class of mild solubilization agents for protein purification. *Biochem J* 305 ( Pt 1) (1995) 337-343.
- [14] M.E. Goldberg, N. Expert-Bezancon, L. Vuillard, T. Rabilloud, Non-detergent sulphobetaines: a new class of molecules that facilitate in vitro protein renaturation. *Fold Des* 1 (1996) 21-27.
- [15] J. Pilon, A. Terrell, P.J. Laybourn, Yeast chromatin reconstitution system using purified yeast core histones and yeast nucleosome assembly protein-1. *Protein Expr Purif* 10 (1997) 132-140.
- [16] K. Ekwall, T. Olsson, B.M. Turner, G. Cranston, R.C. Allshire, Transient inhibition of histone deacetylation alters the structural and functional imprint at fission yeast centromeres. *Cell* 91 (1997) 1021-1032.
- [17] M. Funakoshi, M. Hochstrasser, Small epitope-linker modules for PCR-based C-terminal tagging in *Saccharomyces cerevisiae*. *Yeast* 26 (2009) 185-192.
- [18] R.S. Nozawa, K. Nagao, H.T. Masuda, O. Iwasaki, T. Hirota, N. Nozaki, H. Kimura, C. Obuse, Human POGZ modulates dissociation of HP1alpha from mitotic chromosome arms through Aurora B activation. *Nat Cell Biol* 12 (2010) 719-727.
- [19] B. Li, L. Howe, S. Anderson, J.R. Yates, 3rd, J.L. Workman, The Set2 histone methyltransferase functions through the phosphorylated carboxyl-terminal domain of RNA polymerase II. *J Biol Chem* 278 (2003) 8897-8903.
- [20] N.J. Krogan, M. Kim, A. Tong, A. Golshani, G. Cagney, V. Canadien, D.P. Richards, B.K. Beattie, A. Emili, C. Boone, A. Shilatifard, S. Buratowski, J. Greenblatt, Methylation of histone H3 by Set2 in *Saccharomyces cerevisiae* is linked to transcriptional elongation by RNA polymerase II. *Mol Cell Biol* 23 (2003) 4207-4218.
- [21] T. Xiao, H. Hall, K.O. Kizer, Y. Shibata, M.C. Hall, C.H. Borchers, B.D. Strahl, Phosphorylation of RNA polymerase II CTD regulates H3 methylation in yeast. *Genes Dev* 17 (2003) 654-663.
- [22] H. Goldfine, Use of a filter-paper disk assay in the measurement of lipid biosynthesis. *J Lipid Res* 7 (1966) 146-149.
- [23] C.J. Olbricht, Extracorporeal removal of lipids by dextran sulfate cellulose adsorption. *Artif Organs* 20 (1996) 332-335.
- [24] M. Choder, Rpb4 and Rpb7: subunits of RNA polymerase II and beyond. *Trends Biochem Sci* 29 (2004) 674-681.

- [25] S.M. Orlicky, P.T. Tran, M.H. Sayre, A.M. Edwards, Dissociable Rpb4-Rpb7 subassembly of rna polymerase II binds to single-strand nucleic acid and mediates a post-recruitment step in transcription initiation. *J Biol Chem* 276 (2001) 10097-10102.
- [26] P. Cramer, D.A. Bushnell, J. Fu, A.L. Gnatt, B. Maier-Davis, N.E. Thompson, R.R. Burgess, A.M. Edwards, P.R. David, R.D. Kornberg, Architecture of RNA polymerase II and implications for the transcription mechanism. *Science* 288 (2000) 640-649.
- [27] A.L. Mosley, S.G. Pattenden, M. Carey, S. Venkatesh, J.M. Gilmore, L. Florens, J.L. Workman, M.P. Washburn, Rtr1 is a CTD phosphatase that regulates RNA polymerase II during the transition from serine 5 to serine 2 phosphorylation. *Mol Cell* 34 (2009) 168-178.
- [28] R.A. Young, RNA polymerase II. *Annu Rev Biochem* 60 (1991) 689-715.

## FIGURE LEGENDS

**Figure 1.** Overview of the two methods used for immunoaffinity purification of the Set2 complex from fission yeast. The left side of the flowchart shows the standard centrifugation method and the right side shows the stepwise filtration method. Half of the whole cell extract was centrifuged and half was filtered.

**Figure 2.** Recovery of Set2 and H3 from SS259 (*set2+::FLAG(3)*) fission yeast grown in YES media at 30°C. (A) Immunoblot analyses of FLAG-tagged Set2 and H3 in the total lysate, centrifuged supernatant (Sup.), centrifuged pellet, and filtrated lysate (Sup.). After preparation of whole cell extracts using a BeadBeater, the lysate was processed by centrifugation or consecutive filtration to separate the supernatant and cell debris. Each lane contained 50 µg of total protein. After resolving each sample by SDS-PAGE, Set2-FLAG and histone H3 were probed using an anti-FLAG mouse monoclonal antibody and anti-H3 rabbit polyclonal antibody, respectively. (B) Immunoblot analyses of Set2 in serially diluted total lysate, filtrated lysate, centrifuged supernatant, and centrifuged pellet samples. The initial total protein concentration was 10 µg/µl. The lower left panel shows 8 µl of the total lysate, filtrated lysate and centrifuged supernatant resolved by SDS-PAGE and stained using Coomassie Brilliant Blue as a loading control. The lower right panel shows a quantitative analysis of the raw immunoblot data shown in the upper panel.

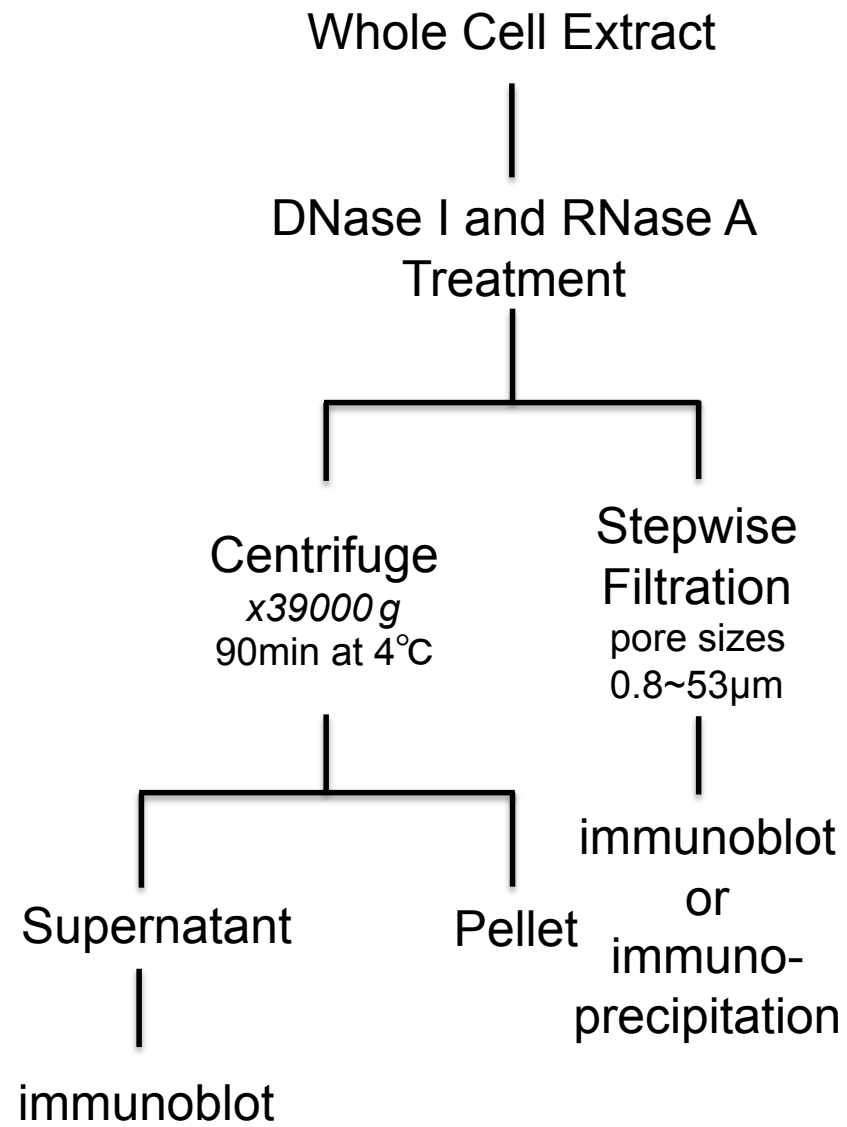
**Figure 3.** The loss of Set2 protein at each stage of the filtration process. Immunoblot analyses of FLAG-tagged Set2 in 5 µl aliquots of the lysate obtained after each filtration step. The raw immunoblot data are shown in the upper panel and the quantified data are shown in the lower panel.

**Figure 4.** Immunoaffinity purification of the Set2 complex from the filtrated lysate and centrifuged supernatant. The FY2002 (wild type) strain was used as the no tag control and the SS297 (Set2-FLAG(3)) strain was used as Set2-FLAG. The anti-FLAG (M2) immunoaffinity-purified Set2 complex was eluted by the addition of 3× FLAG peptide. (A) Comparison of the amounts of FLAG-tagged Set2 recovered from the filtrated lysate and centrifuged supernatant by immunoprecipitation (IP). Total protein (50 µg) was used as the input and 500% or 200% of input was used for anti-FLAG immunoprecipitation. F, filtrated lysate; C, centrifuged supernatant. The raw immunoblot data are shown in the upper panel

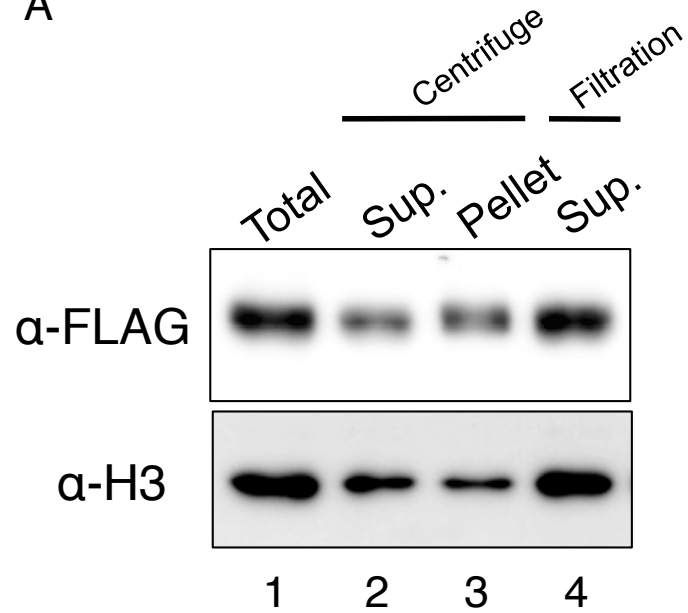
and the quantified data are shown in the lower panel. (B) Immunoblot analysis of Set2-FLAG immunoaffinity purified from the filtered lysate showing full-length Set2-FLAG (arrowhead) and degraded Set2-FLAG derivatives (asterisks). (C) SDS-PAGE and silver staining of the immunoaffinity-purified Set2 and associated proteins from the filtered lysate. The arrowhead indicates full-length Set2-FLAG.

**Table 1.** Proteins in the immunoaffinity-purified Set2-FLAG complex identified by mass spectrometry.

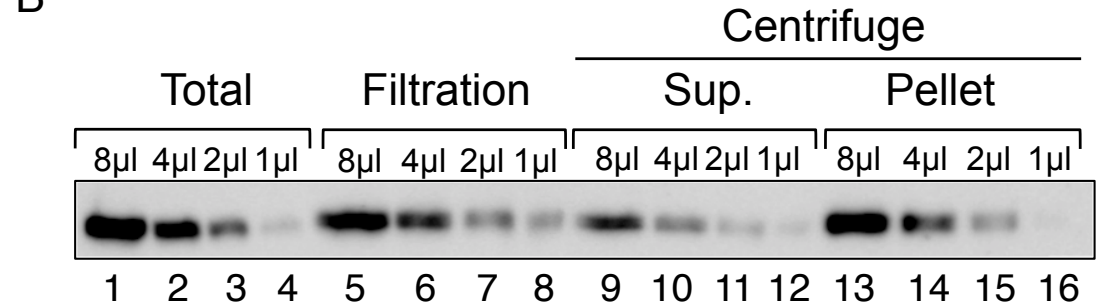




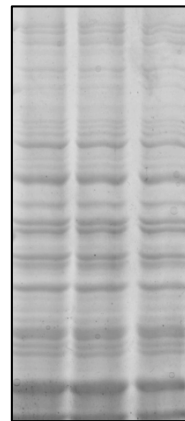
A



B



Total  
Filtration  
Centrifuge  
Sup.



CBB

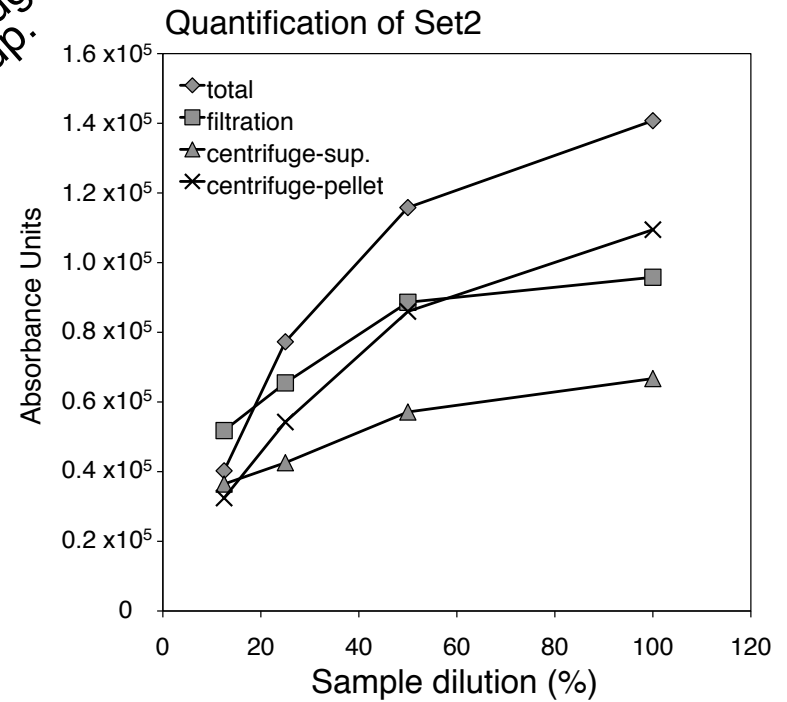


Fig. 3

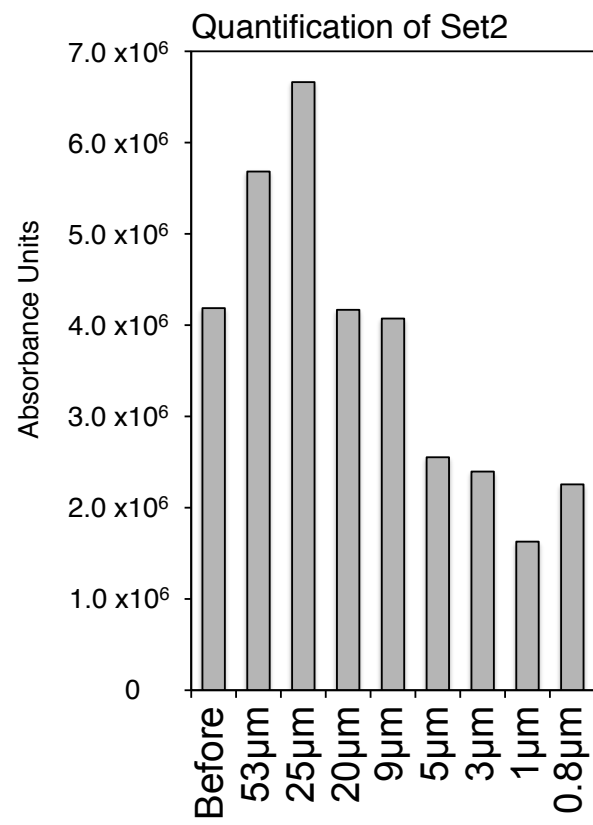
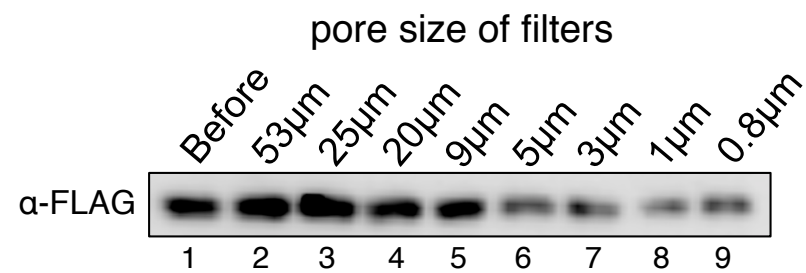


Fig. 4

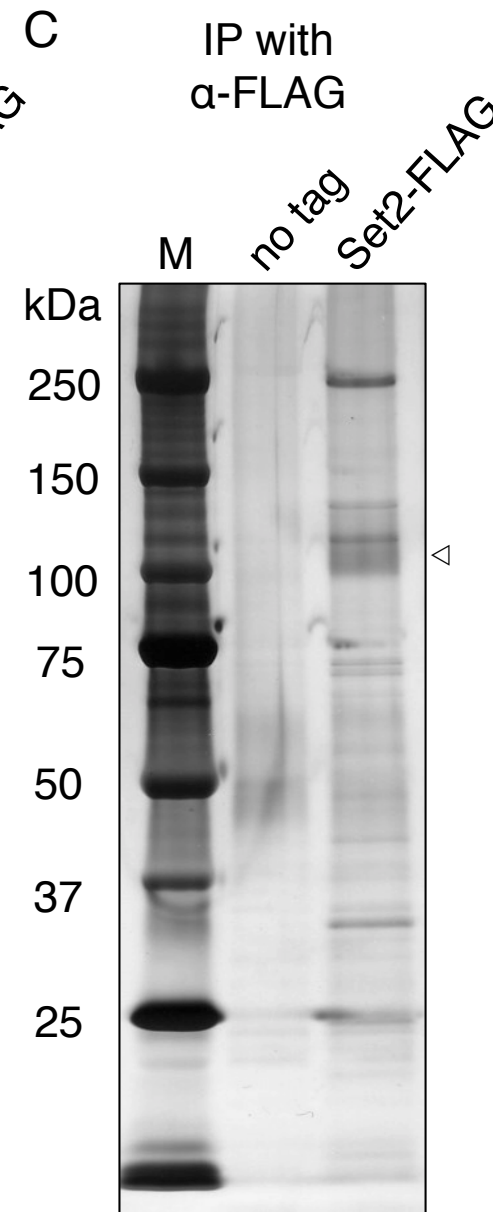
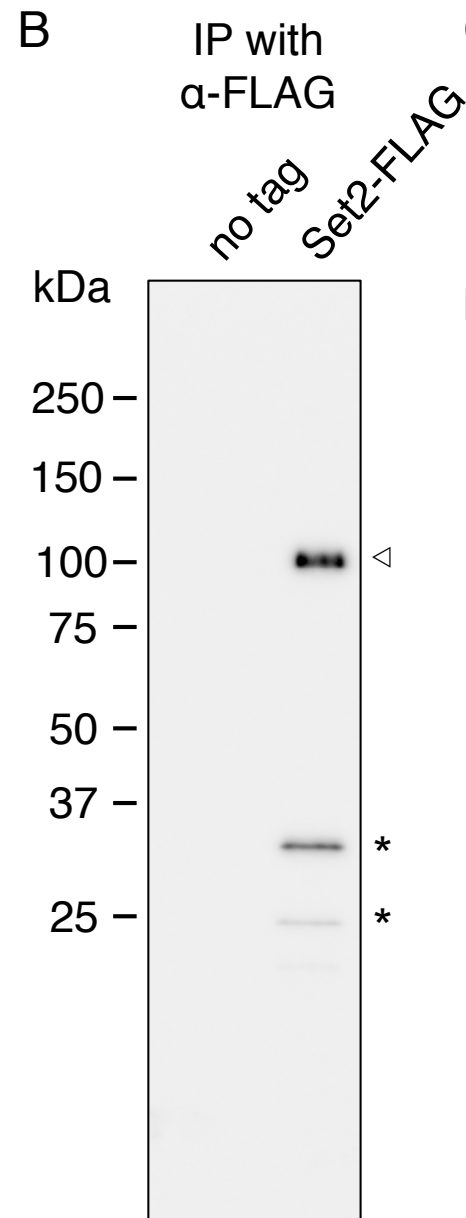
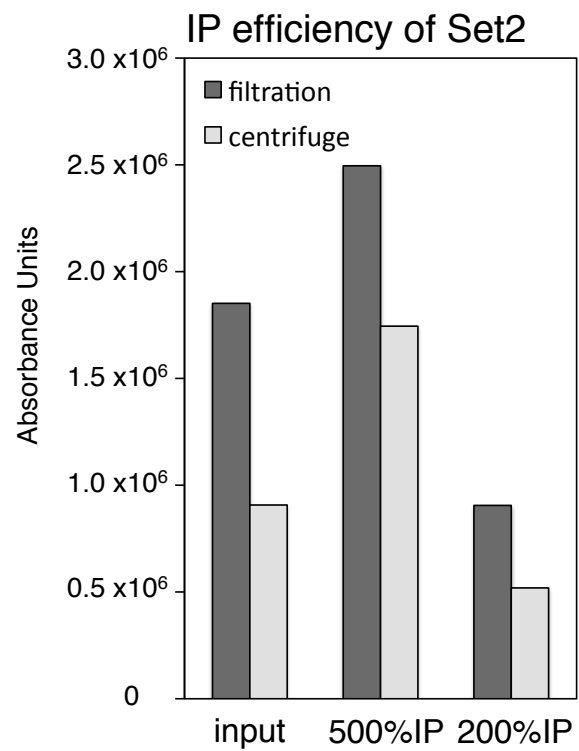
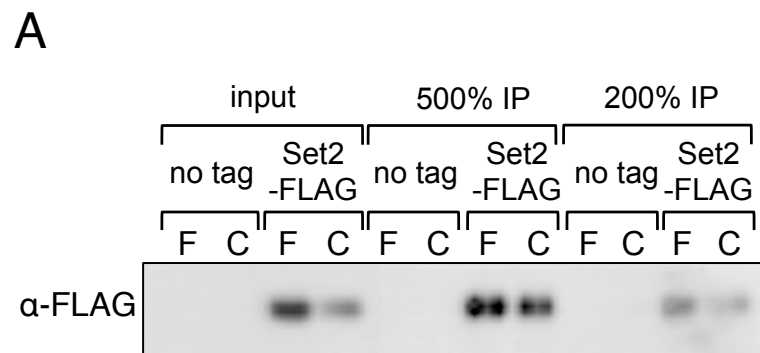


Table 1

identified proteins	peptide counts	
	no tag	Set2-FLAG
Set2	0	119
Rpb1	0	66
Rpb2	0	33
Rpb3	0	9
Rpb4	0	4
Rpb5	0	5
Rpb7	0	1
Rtr1	0	1